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VOLUME I

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

LUNAR RECEIVING LABORATORY

BIOLOGICAL LABORATORY

OPERATIONS PROCEDURE

FOR

APOLLO 11

MANNED SPACECRAFT CENTER
HOUSTON, TEXAS
July 7, 1969

PROJECT DOCUMENT COVER SHEET

LUNAR RECEIVING LABORATORY

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APOLLO 11

REPORT NUMBER	DATE
MSC 00030	July 7, 1969

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1.0 GENERAL SECTION

1.1 BIOPREPARATION

1.1.1 Consolidated Biopreparation Procedures for Lunar Samples

The major objective of the Biopreparation Laboratory is to process (i.e., prepare, weigh, and package) the prime and pooled lunar samples for distribution to the biological test laboratories. Prime lunar samples will consist only of samples collected from depths of 1 to 8 inches below the lunar surface and immediately quarantined in sterile containers. Therefore, neither human nor environmental contamination of the prime sample will have occurred during the collection and subsequent transit to the Lunar Receiving Laboratory (LRL). Conversely, pooled lunar samples will consist of samples collected from various surface sites and placed in the same container(s). The prime samples will be considered sterile; the pooled nonsterile. Homogenates of the prime and pooled lunar sample types will be processed for bioanalyses.

The actual processing of the prime and pooled lunar samples will consist of screening out particles of less than 104-micron diameter, reducing the remaining material to a mean particle diameter of approximately 25 microns, and combining the two (fig. 3.1.1-1, generalized size reduction scheme for lunar samples). This combination will then be statistically split and weighed into aliquots appropriate to the requirements of the biological test procedures. For example, the aliquots required by the Virology Laboratory vary from those required by the Botanical Laboratory.

1.1.2 Direct Observation of Prime and Pooled Lunar Samples

Prime and pooled lunar samples will be examined directly for microorganisms by employing electron microscopy, phase-contrast microscopy, white light microscopy, and fluorescent microscopy, using appropriate staining techniques. For example, dry samples of prime and pooled lunar material will be examined with the compound microscope and the stereomicroscope. Also, aqueous extracts of prime and pooled lunar material will be concentrated

and observed by phase-contrast microscopy, then stained and observed by light microscopy. Dilute suspensions will be observed by electron microscopy.

The preparation of lunar samples and all light-microscopic observations will be performed within the Class III Cabinetry in room 1-126. Then, the lunar samples to be examined by electron microscopy will be passed out and transferred to room 189.

1.2 BACTERIOLOGY AND MYCOLOGY

The objective of the bacteriological and mycological analyses procedure is to identify microorganisms of extraterrestrial origin (if any) present in the prepared prime and pooled lunar samples. No universal medium exists for culturing microorganisms; therefore, prepared prime and pooled lunar samples will be subjected to various culture media. Four different media will be inoculated with prepared prime lunar samples, and seventeen different media will be inoculated with prepared pooled lunar samples. Culture media will be prepared in two different dilutions and will be incubated at four different temperatures and under three different atmospheric conditions. During incubation, the media will be observed for signs of growth. All isolates will be characterized and identified by morphological and biochemical techniques and compared with preflight and postflight cultures of samples from the crewmen and spacecraft. These operations will be performed inside the Class III Cabinetry, room 1-123.

1.3 VIROLOGY

1.3.1 Lunar Sample Toxicity Determination

The objective of the lunar sample toxicity-determination procedure is to determine whether extracts of prime lunar samples are toxic for tissue culture systems. This procedure will be initiated approximately 24 hours prior to beginning the complete virology procedures. Three types of cell cultures, that is, the primary African green monkey kidney (GMK), the primary human embryonic kidney (HEK), and the human embryonic lung (WI-38), will be

exposed to an extract from a sterile prime lunar sample (sample requirements will be 4.014 g; six cell cultures times three cell types times 0-223 gm). The extract will be the supernatant from a 50-percent suspension of lunar material in tryptose phosphate broth containing 0.5 percent gelatin.

For example, three culture tubes of each cell type will contain 1.0 ml of maintenance medium with antibiotics and will be inoculated with 0.2 ml of supernatant from the 50-percent suspension of sterile prime lunar material. The cultures will be examined 2 hours after inoculation, and any required adjustments of pH will be made. In addition, three culture tubes of each cell line, dry monolayers, will be inoculated with 0.2 ml of the supernatant from the 50-percent suspension of lunar material. After a 2-hour incubation at room temperature, the inoculum will be discarded, and each culture will receive 1.0 ml of maintenance medium with antibiotics. The cultures will be incubated at 35° C and will be examined periodically for toxicity.

The tissue cultures required for this toxicity-determination procedure consist of: (1) nine screwcap tube cultures of GMK, (2) nine screwcap tube cultures of HEK, and (3) nine screwcap tube cultures of WI-38.

Notably, if the prime lunar sample proves to be toxic, resulting in the destruction of the tissue cultures, further biotests will not be initiated until a complete chemical analysis of the prime lunar sample has been determined. However, dialysis procedures will be initiated immediately in an attempt to remove the toxicity present in the prime lunar sample. If extracts of the prime lunar sample are not toxic, the complete virology procedures will be conducted as planned.

1.3.2 Virology Procedure

The complete virology procedure consists of three parts.

a. Analysis of the effects of lunar material on tissue cultures and embryonated eggs, that is, lunar material will be introduced into hosts to isolate any

agents capable of replicating. The hosts will consist of six types of cell cultures and 6- and 10-day-old embryonated eggs. The types of lunar material used will consist of prime and pooled lunar samples. Extracts of these samples will be introduced into the eggs as a 50-percent suspension of tryptose phosphate broth and will be introduced into the cell cultures as a supernatant of the suspension. Any agent detected in the hosts will be propagated, studied, and (if possible) identified.

b. Inoculation of mycoplasma media with lunar material. Media capable of supporting mycoplasma growth will be inoculated with prime and pooled samples. Extracts of each sample will be introduced as the supernatant from a 50-percent suspension; the suspending medium will be the broth form of the mycoplasma medium used. Any mycoplasma-like organisms isolated from the lunar samples will be propagated, studied, and (if possible) identified.

c. Characterization of the viral and mycoplasma flora of Apollo crew members. To isolate infectious viral agents present on the crew members, extracts of specimens (e.g., blood, pharyngeal swab, urine, and feces) will be introduced into the medium developed by Chanock, Hayflick, and Barile. Agents isolated will be propagated, studied, and (if possible) identified.

1.4 WARM-BLOODED ANIMALS

1.4.1 Mammalian Vertebrate

The prime objective of analyzing the effects of the lunar sample on a mammalian vertebrate (germ-free mice) is to determine whether the lunar material contains extraterrestrial, biologically active agents capable of producing disease in the germ-free mice. The secondary objective is to determine whether the lunar material contains agents capable of replicating in the test system.

To test lunar material for disease-producing, extraterrestrial, infectious agents in the mouse, the following questions must be answered.

a. Do samples of the lunar material cause death?

b. Do samples of the lunar material cause visible distress or illness?

c. Do in vitro microbiologic examinations of organs or tissues produce organisms not identifiable as terrestrial?

d. Do the classical clinical parameters (i.e., elevated body temperature, inappetence, body weight loss, total leukocyte increase, differential leukocyte count change, drop in hematocrit, change in total serum protein, dramatic drop in the serum albumin fraction) indicate the occurrence of infectious disease processes?

e. Do gross and microscopic post mortem examinations indicate the presence of any infectious process?

The test system, composed of male and female, germ-free, CD-1 strain, random-bred Swiss mice, will consist of one test group and three control groups. The test group will be exposed by intraperitoneal injection of the nonsterile lunar stone or its equivalent. The first control group will be exposed to dry-heat-sterilized lunar soil or its equivalent; the second control group will be exposed to an equal amount of the diluent without the lunar soil complement; the third control group will not be exposed to any foreign material. The same test protocol will be followed using both prime and pooled samples of the lunar material. After inoculation of the mice, the questions previously listed will be answered by completing the following test criteria (see section 3.4, appendixes A to D).

a. Direct visual observation of the test system

b. Microbiological observations — Samples to be cultured will be turned over to the microbiology group for anaerobic and aerobic incubation as well as further processing and work-up.

c. Classical clinical observation

(1) Body temperature

(2) Body weight

(3) Total leukocyte count

(4) Differential leukocyte count

(5) Hematocrit

d. Gross and microscopic post mortem observations

e. Clinical chemistry

(1) Total serum protein

(2) Differential serum proteins

1.4.2 Avian Vertebrate

The effects of the lunar sample on avian vertebrate (specifically, the Japanese quail, Coturnix coturnix) will also be analyzed to determine whether the lunar sample contains agents capable of producing disease or capable of replicating. Only pooled samples of the lunar material will be used for the avian exposure. The test protocol will consist predominately of observations (i.e., watch, wait, and note results), mortality, visible disease, and egg production. If changes are noted in these parameters, Clinical Laboratory tests will be performed. Of course, if no changes are noted, no Clinical Laboratory tests will be performed.

1.5 COLD-BLOODED ANIMALS

The effects of lunar samples on selected invertebrate and fish species will be analyzed to determine whether lunar samples contain biologically active agents capable of either causing disease or replicating in cold-blooded specimens. Eight invertebrate species (the euglenia, paramecium, planaria, German cockroach, domestic housefly,

greater wax moth, brown shrimp, and oyster) and two fish species (the fathead minnow and the killifish) will be exposed to 0.220 gram of pooled lunar sample in an attempt to detect agents capable of interfering with the normal life processes of the host species.

The general contingency procedures for cold-blooded animals are presented in appendixes K through Q in section 3.5.

1.6 LRL BOTANICAL LABORATORY PROGRAM SUMMARY

The Botanical Program was initiated on the recommendation of the U. S. Department of Agriculture to determine if the introduction of lunar materials to the terrestrial biosphere would result in the appearance of an epidemic disease(s) in plants. To accomplish this objective, a program was developed at the MSC Lunar Receiving Laboratory to determine effects of lunar materials on the following challenge systems: algae (4 species), spores and seeds (8 species), seedlings (13 species), and tissue cultures (8 species). Because one species is used in three of the challenge systems, a total of 31 species is challenged. These four challenge systems have been established and maintained in an aseptic environment in which light, temperature, and relative humidity are monitored at regular intervals. In addition, maximum precautions are taken to ensure containment of both lunar materials and challenge systems.

Upon receipt of the lunar materials from the Biopreparation Laboratory, the four challenge systems are treated in the following manner.

a. Algae are challenged with 0.220 gram of lunar material or control material per 20 milliliter of fresh subculture in a test tube.

b. Spores or seeds are germinated in the presence of lunar material or control material by placing 0.220 gram of the material on top of 25 spores or 10 seeds that have been placed previously in a special germination chamber.

c. Seedlings are challenged by rubbing their leaves with a slurry of lunar material or control material buffered

in aqueous media. Each plant is treated with a suspension of 0.220 gram of material in 5 milliliter of phosphate buffer. After abrading the leaves with this slurry, all remaining material is washed onto the roots by using another 5-milliliter portion of buffer.

d. Tissue cultures are challenged by sprinkling 0.220 gram of lunar material or control material over the surface of each culture that is growing on semisolid nutrient medium.

Control materials include terrestrial basalt and lunar matter that have been sterilized by dry heat after being ground and processed in a manner identical to that employed with nonsterile lunar material used for challenging.

After incubation, the challenge systems are kept under constant observation during a 30-day period to determine whether changes take place in the plants treated with lunar material that do not take place in plants challenged with control materials. In addition to routine visual observations of the plants, a system of collecting both qualitative and quantitative data on all challenge systems will be used. These data will be collected at macroscopic, microscopic, ultrastructural, and biochemical levels.

On the basis of the results obtained, the Manager of the Botanical Sample Laboratory will make recommendations to the Test Director of the Biomedical Sample Laboratory concerning the status of the lunar materials as a source of plant pathogens. This recommendation is based on a decision-making process summarized in the lunar-sample-release flow chart, figure 3.6.4-10.

2.0 PREOPERATIONS CHECKLIST

2.1 BIOPREPARATION

2.1.1 Personnel

No personnel list is available.

2.1.2. Equipment

2.1.2.1. General

Item	Quantity
Oven, vacuum	1
Pump, vacuum	1
Tubing, vacuum	1
Filters, Millipore, inline, for air-inlet line	3
Stand, support	1
Funnels	Multiple
Balance, analytical, 100-gram maximum	2
Balance, analytical, 500-gram maximum	1
Spatulas	6
Weighing boats	Multiple
Crusher, sample	1
Grinder, mortar	1
Rulers, metric	3
Beakers, 600 ml	6
Beakers, 400 ml	6
Beakers, 50 ml	6
Cylinders, graduated, 25 ml	6
Sieve assemblies, 104μ	2
Slides, microscope	Multiple
Cover slips	Multiple
Alcohol, n-butyl	200 ml
Canada balsam	10 ml
Sticks, applicator	10
Mixer, hand	1
Petri dishes, glass, 60-mm diameter	4
Brushes, sable	12
Containers, sample	2
Containers, dunk tank	3
Oil, immersion	1 bottle
Riffle samples	--
Labels	100

Item	Quantity
Pencils	6
Pads, writing	2
Base, microscope	1
Tray, slide	1
Micrometer stage	1
Pipettes, Pasteur	40
Pencils, diamond	3
Spoons, measuring	3
Wrenches	6
Pycnometer	1
Foil, aluminum	1 roll
Mirror	1
Container, trash	1
Jars, large	2
Source, AO-light	1
Thermometer	1
Screwdriver	1
Paper, lens	1 book
Water, sterile	500 ml

2.1.2.2 Materials Used Inside Class III Cabinetry

Item	Quantity
Centrifuge, clinical	1
Test tubes, 16 by 125 mm	32
Sterile phosphate buffer	16 tubes (5 ml/tube)
Pipette, Pasteur	2 tubes (18/tube)
Slides, microscope	100
Cover slips	100
Spatulas	4
Petri dishes, glass, 60-mm diameter	4
Gluteraldehyde	100 ml
Mercuric, chloride solution	20 ml
Vials, glass	8
Stoppers	8
Caps, plastic	8
Mixer, Vortex	1
Sources, ultraviolet light	4
Transformers, ultraviolet light	2
Jack, laboratory	1
Reagents, gram stain	1 set
Reagents, acidfast	1 set
Rack, staining	1
Forceps, large	1

Item	Quantity
Forceps, small	2
Hotplate	1
Oil, immersion	1 bottle
Timer	1
Bottles, water	4
Bottle, plastic wash	1
Tray, laboratory technique staining	1
Culture, gram stain control	1
Culture, acidfast stain control	1
Control, fluorescent	1
Propipettes	2
Bulbs, rubber	2
Petri plate, glass, with glass rod	1
Rack, test tube, 40-hole	1
Vaseline	1 bottle
Applicators	10
Sources, AO-Light	2
Bases, microscope	2
Paper, lens	2 books
Filter, ultraviolet for microscope	1
Cellowipes	2 boxes
Container, trash	1
Container, discarded pipette	1

2.2 BACTERIOLOGY AND MYCOLOGY

2.2.1 Personnel

No personnel list is available.

2.2.2 Equipment

2.2.2.1 Equipment for Room 1-125 (West)

Item	Quantity
Incubators	4
Lamp	1
Bags, anaerobic	Multiple
Racks, test tube	Multiple
Pipettes, Pasteur	Multiple
Pipettes, 10 ml	Multiple

Item	Quantity
Propipette	--
Bulb, pipette	1
Pencil, diamond point	1
Pencil, wax	1
Tape, autoclave	--
Tape, masking	--
Microscope	1
Slides, microscope	Multiple
Pan, discard	1
Can, discard	1
Box, trash	1
Indicator, anaerobic	1
Material, gram stain	1
Reagent, biochemical	--
Towels, paper	--
Sponge	1
Towels, cloth	Multiple
Scissors	--
Pack, gas	Multiple
Pencil, felt-tipped	1
Bottle, hypochlorite	1
Bottle, water	1
Mixer, Vortex	1
Hotplate	1
Cel-fibe	--
Vaspar	--
Oil, immersion	--
Microincinerator	1
Loops	Multiple
Paper, filter	--
Cages, animal	Multiple
Stack-a-shelf	Multiple
Forceps	--
Syringe	1
Labels	Multiple

2.2.2.2 Equipment for Room 1-109 (West)

Item	Quantity
Racks, test tube	Multiple
Spreaders, glass	Multiple
Turntables	Multiple
Pipettes, 2 ml	Multiple
Propipettes	Multiple

Item	Quantity
Pans, discard	Multiple
Packs, gas	Multiple
Scissors	--
Forceps	--
Bottle, water	1
Syringe	1
Bags, anaerobic	Multiple
Trays	Multiple
Slides	Multiple
Slide holders	Multiple
Stain, gram	--
Rack, gram stain	1
Pencil, diamond point	--
Pens	Multiple
Pencils	Multiple
Pencil, wax, marking	1
Pipettes, Pasteur, short	Multiple
Pipettes, Pasteur, long	Multiple
Balance	1
Water bath	1
Thermometer, water	1
Hotplate	1
Paper, weighing	--
Paper, filter	--
Mixer, Vortex	1
Grippers	Multiple
Kimwipes	--
Towels, paper	--
Oil, immersion	--
Hypochlorite	--
Loop	Multiple
Microincinerator	1
Bulbs	Multiple
Reagents	Multiple
Vaspar	--
Labels	Multiple
Filter, Millipore	1
Media, Millipore	--
Syringe, Millipore	1
Tube, capillary	--
Beaker, graduated	1
Bottles, sterile	Multiple
Steroscope and light supply	1
Microscope	1
Tape, autoclave	--
Cans, tin	Multiple

Item	Quantity
Shelves	Multiple
Spatulas	Multiple
Sponges	Multiple
Caps, sterile	Multiple
Petri plates, sterile	Multiple
Lamps	Multiple

2.2.2.3 Equipment for Room 122 (West)

Item	Quantity
Centifuge	1
Microscope, dissecting; and light source	1
Microscope, compound; and camera	1
Burners, incinerator	3
Lamp	1
Jack, dissecting microscope	1
Pans, discard	4
Tongs	--
Variety of loops, needles, and so forth	--
Slides, microscope	Multiple
Cover slips	Multiple
Cotton, lactopheonol, blue	--
Rack, 48-hole	--
Pipettes, 1 ml	1 can
Propipettes	2
Pipettes, Pasteur	75 (approximately)
Oil, immersion	--
Markers	Multiple
Labels	Multiple
Swabs	Multiple
Spreaders	30
Cover slips, sterile	Multiple
Petri dish	1
Forceps, large	--
Forceps, small	--
Towels, paper	--
Stains, Ascospores	--
Media	--

2.3 VIROLOGY

2.3.1 Personnel

2.3.1.1 Number

a. Maximum number in all virology laboratories:

Position	Staff
Technical staff	6
BRN supervisor	1
NASA virologists	2
Consultants	2
Total	11

b. Maximum number in the individual laboratories:

Room	Staff
105	4
107	9
109	3

c. Apparent discrepancy: The apparent discrepancy results from the cross use of personnel.

2.3.1.2 Organizational Relationships

The organizational relationships of the Virology Laboratory personnel are shown in the data flow plan, figure 2.3.1-1.

2.3.2 Equipment

2.3.2.1 Equipment for Room 105

a. General equipment:

Item	Quantity
Autoclave	1
Microscope	1

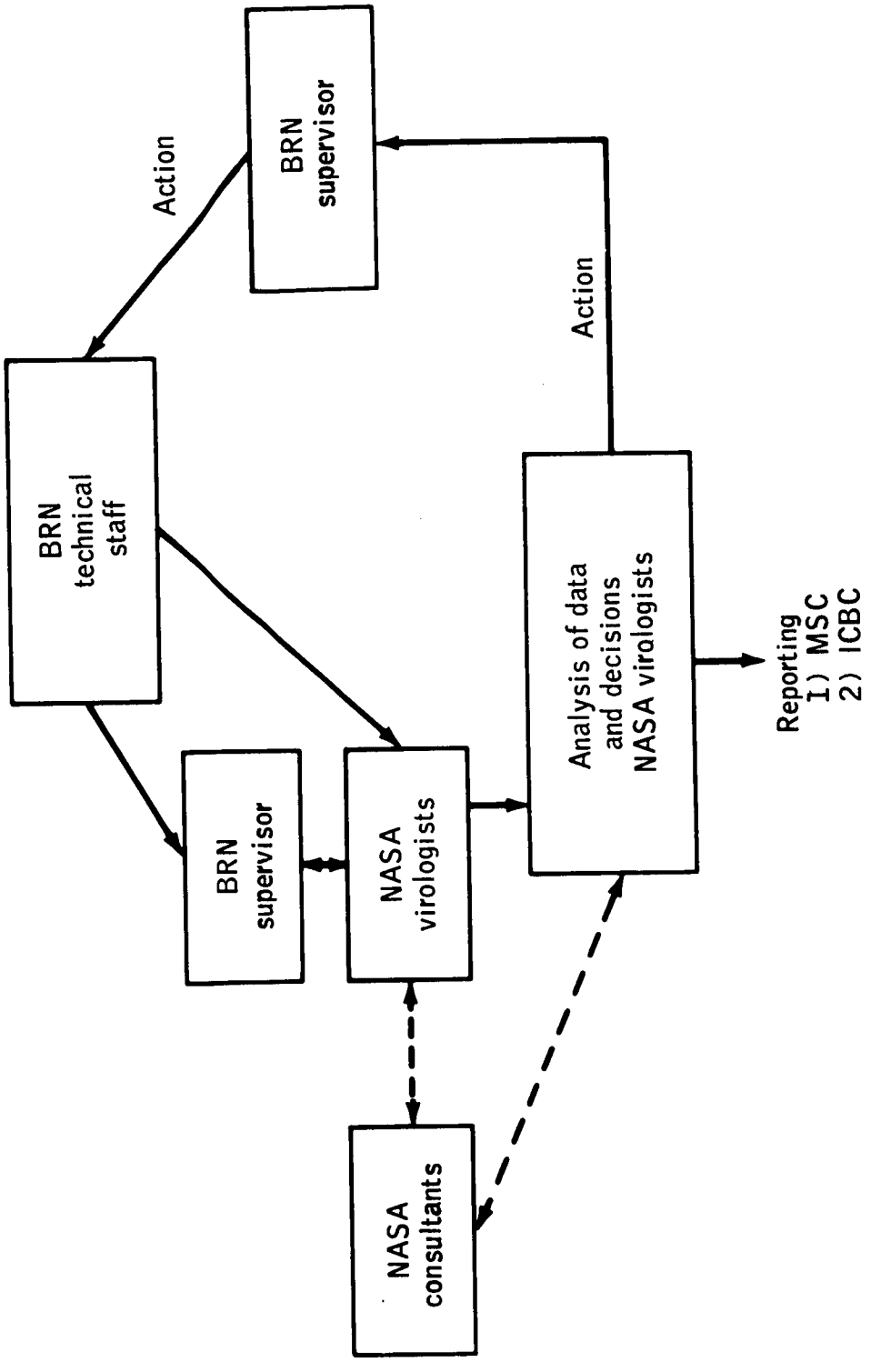


Figure 2.3.1-1.- Data flow plan for the Virology Laboratory.

Item	Quantity
Refrigerator	1
Incubators, 37° C	2
Incubator, 37° C, with airtight container	1
Chaicolcentrifuge, with bucket head for 50-ml tubes and adapters	1
Containers, waste	2
Container of phenol disinfectant	1

b. Sample preparation equipment:

Item	Quantity
Forceps	3
Rubber policemen	3
Tubes, centrifuge, 50 ml	6
Pipettes, 1 ml	10
Pipettes, 2 ml	10
Pipettes, 5 ml	10
Vials, 10 ml	6 doz
Tryptose phosphate broth	200 ml
Trypsin inhibitor	20 ml
Aliquots of antibiotics (penicillin, streptomycin, and mycostatin), 2 ml	2
Aliquots of antibiotics (penicillin, staphcillin 6, and mycostatin), 1 ml	2

c. Mice equipment:

Item	Quantity
Syringes, 1-ml	12
Needles, 5/8 in., 26 gage	12
Bottles, water	10
Water, 2-week supply	--
Food, 2-week supply	--
Bedding, 2-week supply	--
Mortar and pestles	1 doz
Scissors and forceps	1 doz

d. Egg equipment:

Item	Quantity
Candles	--
Punch, short point	--
Punch, long point	--

Item	Quantity
Punch, harvest	--
Rack, egg	--
Brush, paint	1
Forceps	6
Mortar and pestle and Alundum	12
Thermometer	1
Hygrometer	1
Incinerator	1
Propipette	1
Bulb, rubber, small	1
Scissors, pointed	1
Needles, 1-1/2 in., 22 gage	12
Syringes, 2-1/2 ml	12
Pipettes, 1 ml	12
Pipettes, 2 ml	12
Pipettes, 5 ml	12
Saline	--
Gelatin	--
Diluent, Veronal buffered	--
Equipment for microtiters	--

e. Mycoplasma equipment:

Item	Quantity
Pipettes, capillary	5 doz
Bulbs, suction	3
Vials, sample storage	4 doz
Cylinder of CO_2	1

2.3.2.2 Equipment for Room 107

a. Crew virology - tissue culture equipment:

Item	Quantity
Microscopes	2
Incubators, 35° C, with roller drum	2
Incubator, 33° C, with roller drum	1
Forma coldfinger	1
Mixer, Vortex	1
Stirrer, magnetic	1
Container of phenol disinfectant	1
Pipettes, 1 ml	50

Item	Quantity
Pipettes, 2 ml	50
Pipettes, 5 ml	50
Pipettes, cornwall, 1 or 2 ml	5
Propipettes	2
Bulbs, rubber	2
Propipette, German, 1 ml	2
Propipette, German, 2 ml	2
Propipettes, German, 5 ml	2
Cellowipes	3 boxes
Containers, waste	2

b. Microtiter equipment:

Item	Quantity
Plates	4 pkgs.
Diluters	12
Droppers and needles	24

2.3.2.3 Equipment for Room 107

a. Lunar soil tissue culture equipment:

Item	Quantity
Autoclave	1
Microscopes	2
Centrifuge, with 50-ml head and adapters	1
Mixer, Vortex	1
Bases, magnetic stirrers	2
Incubator, 35° C	1
Incubator, 30° C	1
Tubes, centrifuge, 50 ml	4
Tubes, test, 10 ml	8
Pipettes, 1 and 2 ml	4 doz
Pipettes, cornwall	8
Tryptose phosphate broth, with 0.5 percent gelatin	200 ml
Aliquots of antibiotics (penicillin, streptomycin, and mycostatin), 2 ml	2
Aliquots of antibiotics (penicillin, streptomycin, and mycostatin), 1 ml	2

b. Mycoplasma and fish tissue culture equipment:

Item	Quantity
Microscope	1
Water, bath	1
Pipettes, capillary	2 doz
Bulbs, suction	2
Forma microtemp, model 70	1
Aquarium	1
Container for tissue culture	1
Pipettes, 1, 2, and 5 ml	2 doz

2.3.2.4 Equipment for Room 109 (Lunar Soil Virology - Eggs)

The equipment for room 109 will be the same as the egg equipment in room 105.

2.4 WARM-BLOODED ANIMALS

2.4.1 Personnel

2.4.1.1 Mammalian Vertebrate

Norman D. Jones, D.V.M., M.S., NASA/USAF, Supervisor, warm-blooded animal experimentation, Veterinary pathologist

Russell E. Stullken, M.S., NASA temporary, Zoologist

Alan J. Kenyon, D.V.M., Ph.D., NASA/University of Connecticut, Consultant, Veterinary biochemist

M. G. Hanna, Jr., Ph.D., NASA/ORNL, Consultant, Experimental pathologist

Theona Vyvial, NASA/University of Texas, Consultant's technician, Immunology

E. Landrum Young, M.S., BRN Supervisor, warm-blooded animal experimentation, Zoologist

Bartrum S. Roark, BRN, Zoology technician

Heather A. Owens, BRN, Clinical Laboratory technician

Beverly J. Tillman, BRN, Histology technician

Patricia A. Noble, BRN, Histology technician

C. David Cost, BRN, Electron microscopy technician

Robert W. Fayle, M.S., BRN, Microbiology technician

Cheeyla Tuchman, BRN, borrowed from CRA during critical period, Clinical Laboratory technician

2.4.1.2 Avian Vertebrate

No personnel list is available.

2.4.2 Equipment

2.4.2.1 Mammalian Vertebrate

a. Animal Biochemistry Laboratory (room 1-128):

Item	Quantity
Autoclave, Ansco, modified, attached to cabinet line	1
Freezer, Delminico (BRN 3922)	1
Oven, Boekel	1
Balance, triple beam, Ohaus (Maintenance ID 000934)	1
Balances, Sartorius (BRN 2929 and 2932)	2
Centrifuge microcapillary, model MB with head for 1-ml test tubes, International (BRN 1780)(Maint. ID 000908)	1
Centrifuge, microcapillary, model MB, with microcapillary head, International (BRN 3830)	1
Centrifuge, "Microfuge" model 152, Beckman (BRN 4156)	1
Reader, microcapillary, circular, revolving International	1
Microscope, "Microstar," with rheostat, mounted AO-Spencer (BRN 3783)	1
Microscope, "Zoom" binocular with rheostat, Bausch and Lomb (NASA 56867)	1
Slide stainer, "Hema-tek," Ames (NASA 73396)	1

Item	Quantity
Spectrophotometer, "Eskalab Alpha," Smith Kline Instruments	1
Refrigerator, "Refrigerette," modified, Sears	1
Refrigerator, "Coldspot 15," Sears (BRN 4044)	1
Power supply, "Duostat," with electrophoresis cells, Beckman	1
Power supply, "Titan-Stat-2," with electrophoresis cells, Johnson-Helena (BRN 3814)	1
Power supply, Hyland	1
Telethermometer and probe, Yellow Springs Instrument (BRN 3332)	2
Biochemistry system, automated, "Clinomak," Laboratory-Line (NASA 75270)	1
Cuvette caroussel washer, "Lavo-mak," Lab-Line (NASA 75271)	1
Densitometer, "Phoroscope," Millipore (NASA 76087)	1
Particle counter, "Celloscope," particle data (NASA 75244)	1
Camera, CU-5 Close-up Land, Polaroid (NASA 76138)	1
Stirrer, magnetic, "Magnistir," Matheson Instruments (Maintenance ID 002284)	1
pH meter, model 10, Corning Scientific Instruments (BRN 3499)	multiple
Supplies, assorted necropsy, hematology, and biochemistry	1
b. Animal Support Laboratory (room 1-127):	
Balance, triple beam, Ohaus	1
c. Animal Support and Microbiology Laboratory (room 1-125):	
Balance, triple beam, Ohaus	1
d. Histology Laboratory (room 147):	
Microtomes, model 820, AO-Spencer (NASA 28470 and BRN 2065)	2
Tissue processer, "Autotechnicon Ultra," Technicon Corp.	1
Tissue processer, "Tissuematon," Fisher (BRN 1567)	1

Item	Quantity
Balance, triple beam, Ohaus	1
Dispenser, Paraffin Barnstead (BRN 1740)	1
Oven, model 2, Precision (BRN 1746)	1
Knife sharpener, microtome, AO-Spencer (BRN 1741)	1
"Frigi-tray," Lipshaw (BRN 3955)	1
Water bath, Lab-Line (BRN 1743)	1
Water bath, Lipshaw (BRN 3962)	1
Refrigerator, Dillon-Lilly (BRN 2244)	1
Microscope, "Zoom" binocular with rheostat, Bausch and Lomb (NASA 59020)	1
Supplies, assorted necropsy and histology	Multiple

e. Veterinary Pathology Office (room 157):

Item	Quantity
Microscope, "Microstar" with rheostat, AO-Spencer (BRN 2707)	1
Camera, microscope, Nikon	1
Microscope, "Photomicroscope" with rheostat, Carl Zeiss (BRN 2799)	1

f. Electron-Microscopy Laboratory (rooms 189, 189E, 189F):

Item	Quantity
Microscope, electron, model 1-A, with power supply, Seimens	1
Ultramicrotome, MT-2, Porter-Blum (BRN 2493)	1
Pumping unit, high vacuum, Balzars (BRN 2633)	1
Hotplate	1
Plate, warming (BRN 1779)	1
Cleaner, automatic, Sonogen (NASA 64844)	1
Water filter and cooler (NASA 59332)	1
Supplies, assorted	Multiple

2.4.2.2 Avian Vertebrate

The following is a list of equipment for use in rooms 1-122 and 1-127.

Item	Quantity
Microscope, compound	1
Microscope, dissecting	1

Item	Quantity
Equipment, dissecting	Multiple
Forceps	
Scissors	
Needles	
Pins, holding	
Containers, to receive tissues and blood for transfer outside of cabinetry	Multiple
Bouin's solution	--
Pencils, marking	Multiple
Forms	Multiple
Paper, writing	
Needles and syringes	Assorted
Camera, Polaroid or 35 mm	

2.5 COLD-BLOODED ANIMALS

2.5.1 Personnel

No personnel list is available.

2.5.2 Equipment

2.5.2.1 General Equipment

Item	Quantity
Microscope, compound	1
Microscope, stereoscopic	1
Balance, triple beam	1
Hach Portable Engineer's Water Quality Laboratory	
Microapplicator	1
Shakers, rotary fermentation	2
Air pumps, aquarium, piston-type	2
Meter, total solids	1
Meter, pH	1
Monitors, field oxygen	2
Thermistors, liquid and air temperature	24
Hygrometer unit	1

Item	Quantity
Microdissection kit	1
Carts, aquarium, Plexiglas	18
Boxes, humidity, Plexiglas, mobile	4
Rack, culture tube, mobile	1
Vacuum unit, aquarium	1
Grippers, mechanical, two types	12
Lights, microscope, gooseneck	2
Buckets, stainless, 15 qt	2
Equipment, miscellaneous dissection	Multiple
Air valves, brass, two- and three-way	36
Lamp and fuel, alcohol	1

2.5.2.2 Supplies

Item	Quantity
Nets, aquarium, nylon, 2- by 4-in.	18
Scrubbers, aquarium, foam rubber	18
Sponges, rubber	10
Bags, plastic "zip lip"	3 doz
Vials, screwcap "dose"	1 gross
Containers, water, plastic, 2-1/2 gal	24
Boxes, trash	4
Labels, paper, gummed for tissue fixatives	Multiple
Bouin's solution	1 pt
FAA	1 pt
Bouin-Dubosque	1 pt
Towel, paper, various	Multiple
Clothes, cloth wipe, various	Multiple
Tongue depressors, large, wooden	Multiple
Pipettes, disposable, 1, 2, 5 and 10 ml	2 doz
Propipettes, various colors	6
Pencils, marking	Multiple
Pan, dissection	1
Vital stains, 1-oz bottles	Multiple
Slides, microscope	Multiple
Cover slips, microscope	Multiple
Rings, whole mount, various	Multiple
Mountant, CMC water miscible	1 jar
Slide boxes (10 slides/box)	5
Tubes, thioglycollate, various	Multiple
Light bulbs, microscope	6
Light puller	1
Stain, Lugol's	100 cc

Item	Quantity
Tubing, Tygon, 1/8-in. i.d.	50 ft
Containers, various	Multiple
Staining supplies	
Glassware, various	Multiple
Bottles, dropping	4
Nigrosin	1 bottle
Set, gram stain	1
Set, staining rack and tray	1
Loops, microbiological streaking	2
Slide boxes (50 slides/box)	4

2.6 BOTANY

2.6.1 Personnel

Dr. C. H. Walkinshaw, Jr., Ph.D., NASA coordinator, Ultrastructure, pathology

Dr. J. A. Vozzo, Ph.D., Alternate NASA coordinator, Mycorrhizae

Dr. H. C. Sweet, Ph.D., BRN supervisor, Physiology, environmental effects and control

Mrs. Carol L. Brezina, M.A., BRN research assistant, Seedlings, seed germination

Mr. W. H. Horne, B.S., BRN research analyst, Tissue culture

Mrs. Mary R. Capezza, B.S., BRN research assistant, Algae

Mr. W. Bolton, BRN laboratory technician, Media preparation, general laboratory work

Dr. S. Venketeswaran, Ph.D., Outside contractor, University of Houston, Tissue culture

Dr. P. Mahlberg, Ph.D., Outside contractor, University of Indiana, Histology

Dr. W. T. Jackson, Ph.D., Dartmouth College, Consultant, Physiology and cytology

Dr. T. W. Tibbitts, Ph.D., University of Wisconsin, Consultant, Physiology

Dr. F. T. Bonner, Ph.D., USDA, Advisor, Physiology

Dr. H. L. Keil, Ph.D., USDA, Advisor, Bacterial plant pathogens

Dr. P. R. Miller, Ph.D., USDA, Advisor, Diagnostic phytopathology

Dr. R. H. Steere, Ph.D., USDA, Advisor, Virology

2.6.2 Equipment

2.6.2.1 Equipment for Room 186

Item	Quantity
Balance, top loading, model P 1000, Mettler	1
Balance, analytical, single pan, model 2403, Sartorious (BRN 2930)	1
Benchtops, Colorlith (4 by 28 ft and 6 by 28 ft), ESCH and Assoc., Inc.	2
Blender, model 1088, on loan, bacteriology, Waring (BRN 1285)	1
Cabinets, floor module, 36 by 48 in. (Curtin)	--
Cabinet, floor module, 30 by 48 in. (Curtin)	--
Cabinet, wall, open faced (Curtin)	--
Cabinet, sliding glass (Curtin)	--
Camera, photomicroscope, color-snap 35, Kodak (NASA 56871)	1
Cart (Curtin)	1
Chair (Curtin)	--
Desks (GSA)	--
File cabinets (GSA)	--
Dictaphone, model 126260, type TB 7-30, Dictaphone Co.	1
Digital read-out, model AT 20, Bausch and Lomb (BRN 2438)	1
Digital read-out, model 41, for use with spectrophotometer, Perkin Elmer (NASA 74080)	1
Sterilizer, dry, Thermolyne, Matheson Scientific (NASA 65247, DS9525m)	1

Item	Quantity
Microscope, electron, model HS8, Hitachi (NASA 74049) (Accelerating voltage, 25 kV and 50 kV; photographic plate size, 75 by 50 mm for double exposure and 75 by 80 mm for single exposure)	1
Fan, Robbin and Myer, Inc.	1
Gas mask, mask no. 84895, clearvue type C, Mine Safety Appliances (supersize back mounted, completion case with two canisters, GME-55 no. M06) (BRN 3975)	1
Regulator, gas pressure, used with algae, on loan from Dr. Silver, Matheson (BRN)	1
Growth chamber, model 1-36L, 8 light boxes/unit, Percival (NASA 73118)	1
Growth chamber, model 37 14, Sherer Gillette (NASA P14746)	1
Humidifier, model 242, Hankscraft (BRN)	1
Hygro-thermograph, model 594, Bendix Co. (NASA P14542)	1
Kjeldak digestion unit, model A, Lab Con (115 volts; 1 phase, 1.2 kW maximum, 60 cycles)	1
Laminar flow, 53 by 33 by 41 in., on loan from Dr. Silver (BRN) (Maintenance ID 002315)	1
Manifold, for algae, BRN constructed	1
Microscope, Bausch and Lomb (NASA 56870)	1
Stirrer, multimagnetic, no. 1269, Lab Line (NASA 70614)	1
Meter, pH, model 7, Corning	1
Pressure can, Millipore, 1-gal. capacity (BRN)	1
Recorder, model SR, no. S-72180, potentiometric, selective range, three speed, Sargent (BRN 3786)	1
Refrigerator, 33 by 32 by 65 in., with mini-freezer, Frigidaire	1
Shaker, gyratory, G-33-10-125, 25-flask, 125-ml capacity, New Brunsurek Sci., Co.	1
Spectrophotometer, model 139, Hitachi Perkins-Elmer (NASA 74040)	1
Table, 4-ft long, Curtin	1
Table, 6-ft long, Curtin	1
Pump, vacuum serial no. 67 366434, model no. 0406-V2A-153E, (Mathesson)	1
Vibrograver, model V-73, Burgess	1

Item	Quantity
Vortex mixer, cat no. 60100-05, Matheson Sci. (NASA)	1
Water Bath, Ultek model no. 390911 7-gal capacity, refrigerated and heated bath and circulation used with electron microscope, Forma Sci., Co.	1
Chiller, water bath, model 2523, on loan from Dr. Silver, Forma Sci. Co.	1
Pump, water circulating, on loan from Allison, Coleman-Rupp Ind.	1

2.6.2.2 Equipment for Room 1-104

Item	Quantity
Air compressor air source for algae, 1.0-cmm approximate maximum capacity Wide, Inc. (Unit complete with Lovejoy coupling, 1/4-hr, open-drip-type motor, pressure switch set for 10 psi minimum and 20 psi maximum, 0- to 2-micron watts filter with automatic drain valve, and regulator with 0- to 5-psi spring to provide 0.05-psi downstream control between 2.5 and 3.5 psi)	1
Air compressor, portable, DeVilbiss type 501, (BRN)	-
Balance, analytical, single pan, semimicro, type H/GT, Mettler	1
Camera, Beseler Topcom (Camera body, super D, cat. no. Bt-301; macrolens, 135-mm, cat. no. Bt-396; extension bellows model III and rail, cat. no. Bt-44; focusing extension tube, cat. no. Bt-82; macrolens reverse adapter ring, cat. no. Bt-397; double cable release, cat. no. Bt-16, no. 5; focusing screen, cat. no. Bt-92; extension tube set, cat. no. Bt-11)	1
Camera, automatic microflex, AFM-B Nikon (consisting of main body, control unit, projection lens, ocular view finder, viewing screen, universal eyepiece adapter, and adapter back for camera back M-35S, (with full 35-mm and 1/2-frame capability)	1

Item	Quantity
Circulating water pump/temperature regulator, type FE, used with Yellow Springs Instruments oxygen meter, Haake (NASA)	1
Recorder, humidity and temperature, model 15-4050E, Hydrodynamics Inc.	
Incubator, bacteria, for incubating Petri dishes, Scientific Industries, Inc. (H2-PI portable incubator canopy, 1-in. thick, solid-core canopy base, shelf unit)	1
Incubator, model 844, with lights, Lab Line Biotronette, Mark II (NASA 72251, NASA 72252)	1
Infrared analyzer, nondispensive, model IR-415A Beckman (part no. 190102, explosion-proof sampler for ease of gas sterilization for monitoring CO_2 0-600 ppm, Beckman model Z-300 pump, part no. 113664)(BRN 3890)	1
Junction box, 12 position, model 4002, Yellow Springs Instruments (NASA)	1
Microscope, phase star, American Optical (BRN 2959)	1
Microscope, "Microstar," series IOT, A.D., American Optical (BRN 3787)(Trinocular rotatable body, ungraduated, mechanical stage, quadruple nosepiece, 10X wide field eyepieces, with objectives 4X, 10X, 45X, and 100X)	1
Monostand, RDA, standard model 6MS 6 ft with model S6-4 Sampson-geared still head, Southwestern Camera Co.	1
Monitor, oxygen, model 53, Yellow Springs Instruments - (includes a four-place sample bath with external thermostatic circulator, magnetic stirring, amplifier unit and two probe assemblies)(NASA)	1
Powerstat, to control light intensity source for photosynthetic assays, Curtin (cat. no. 21740 transformer, variable power stat., Serial 116B-120V/50-60 cycles ac, Superior Electric Co.)(BRN)	1
Tank pressure, 3-gal capacity, Millipore (BRN)	1
Pump, air, Model 2-300, explosion-proof, Beckman	1
Pump, water, variable speed, HZ1R031, Cole-Parmer (BRN)	1

Item	Quantity
Recorder, electronic strip chart, model 2550-311-100-100-100-10, (no. of pens, 3; input, 0 to 1 volt)	1
Recorder, model 80, single channel, Yellow Springs Instruments (NASA 75307)	1
Projector, 800 slide, no. 8815, Kodak Carousel	1
Spectroradiometer, range 380 to 1050 nm, model SR, ISCO (including remote probes)(BRN 3818, BRN 3817)	1
Tables, stainless steel, 36 by 16 by 36 in., no. 999-995 Curtin	1
Read-out thermistor, model 43tD, single-channel input, Yellow Springs Instruments (NASA)	1
Tool chest, 29 by 33 by 20 in., cat. no. 873-880, Curtin (BRN 3801, BRN 4288)	1
Cleaner, vacuum micromatic, Kent	1
Water bath, for O_2 meter, model 5301, Yellow Spring Instruments (NASA)	1
Water bath, media to maintain agar in liquid state at 50° C. Lab Line (Serological-utility bath, ambient to 60° C with an accuracy of ±0.1°, total range ± ambient 90° C, inside dimensions approximately 13 by 10 by 7 in.; Stainless steel with polished stainless-steel cover for 110 to 120 volts, 50/60 cpm) (BRM 3897)	1
Water circulator, automatic thermal unit to regulate water bath temperature from 50° to 100° F (pump delivers 6 gal/min, 115 volts, 60 cycles) Frigidheat Industries	1

2.6.2.3 Equipment for building 262, Room 108

Item	Quantity
Balance, microtorque, model DR-100 Brinkman (basic scale range, 0 to 100 mg; direct digital readability of 0.1 mg; accuracy of ±0.1 mg)(BRN 3880)	1
Glove box, bacteriological, 36 by 29 by 36 in., series 9185, Lab Con Co. (NASA 73696)	1
Growth chamber, model 1-36L, 8 light boxes/unit, Percival (NASA 73117, BRN 3264)	1
Hygro-thermograph, model no. 594, Bendix Co. (NASA P14539, NASA P14540, NASA P14541)	1

Item	Quantity
Infrared analyzer, model 415A, nondispersive, with explosion-proof sampler, Beckman (BRN)	1
Integrator, electronic, integraph model 49A	1
Analyzer, oxygen, model 778, on loan from Lynn Houser, BRN building 32, Beckman	1
Microscopes, photo, (stand with tube head and optovar, and the following: electric exposure, binocular body, stage carrier, nosepiece, substage, lamp, achromaticaplantic phase contrast condenser, eyepieces or planapochromat objectives)(NASA 74114)	--
Picoammeter, model 417, high-speed, Keithley Instruments, Inc. (range 10^{-13} ampere full scale to 3 by 10^{-5} ampere, accuracy ±3 percent of full scale; zero drift of less than 1 percent of scale per 8 hours on any scale; recorder output of ±3 volts at up to 1 milliampere for full-scale meter deflection; for 110 to 120 volts.)	1
Recorder, model 43, to be used with integraph, photovolt linear/log varicord, Keithley (BRN 3875)	1
Recorder, model 80, Yellow Springs Instruments (NASA 75451)	1
Shaker, incubator, cat. no. 3595-2, Matheson Scientific (NASA 66262)	1
Read-out thermistor, model 43TD, single channel input, Yellow Springs Instruments (NASA)	1
Water bath, cat. no. 65320-10, stainless steel with cover, Matheson Lab Line, (ambient to 60° C with 0.1° accuracy)(BRN 3896)	1

3.0 MISSION PROCEDURES

3.1 BIOPREPARATION

3.1.1 Consolidated Biopreparation Procedures for Lunar Samples

3.1.1.1 Objectives

Prepare, weigh, and package prime and pooled lunar samples for distribution to individual biological laboratories.

3.1.1.2 Distribution Protocol

a. Test design — Lunar samples will be received in the Class III Cabinetry in room 1-126 (Biopreparation Laboratory) from the Vacuum and Transfer Laboratory. Samples will be of two types: those collected under sterile conditions (prime sample) and those collected under nonsterile conditions (pooled sample). Homogenates of each sample will be prepared for bioanalyses. The test design is presented in figure 3.1.1-1.

Sample processing will consist of screening out particles less than 104μ in diameter, reducing the remainder to a volume median diameter (VMD) of approximately 25μ and pooling the two. This combination will then be split statistically and weighed into aliquots appropriate to the biological protocols. Samples will be packaged and transferred out of the cabinets in room 1-126 to the various laboratories for the conduct of experiments.

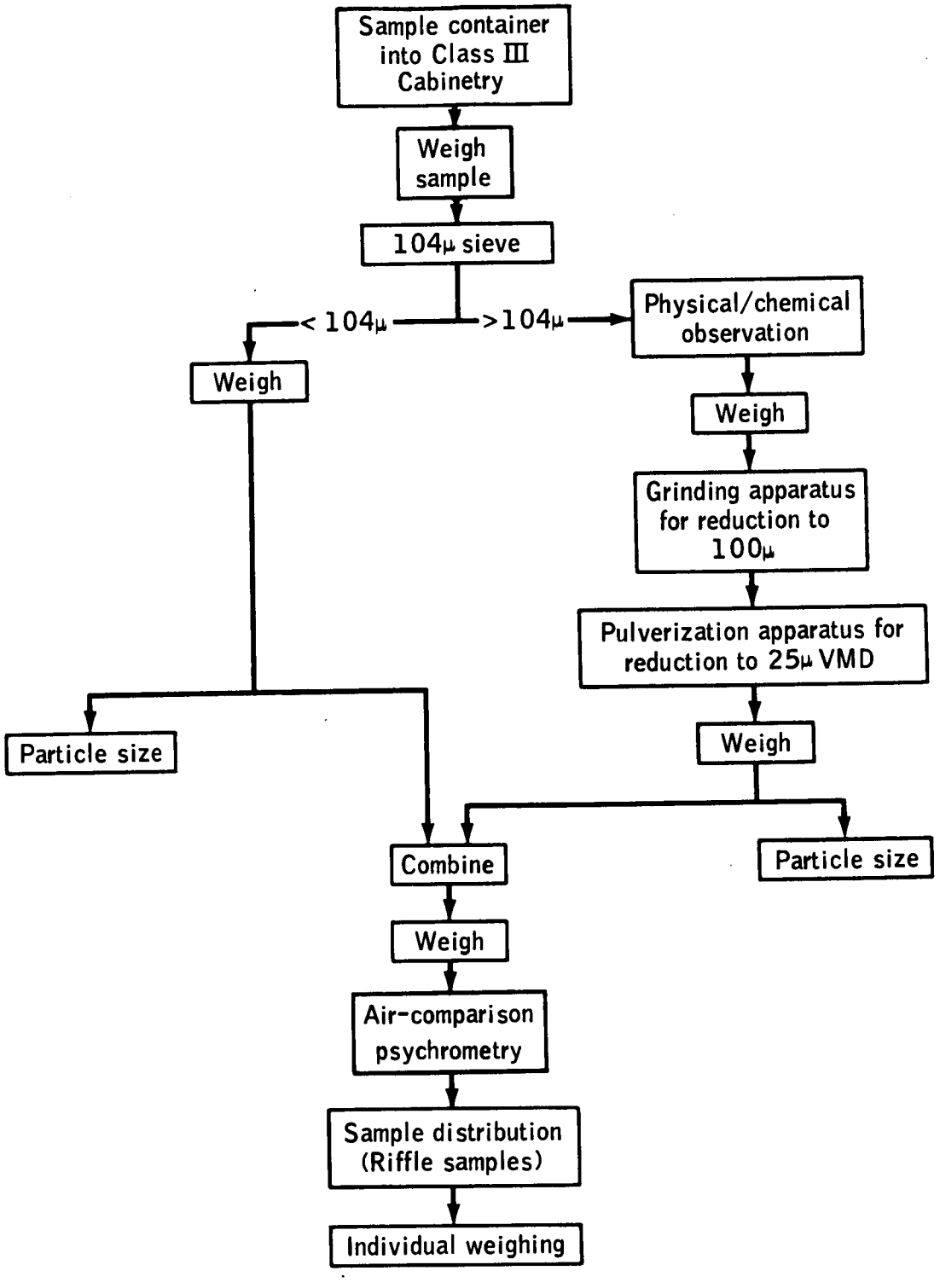


Figure 3.1.1-1.- Generalized size-reduction scheme for lunar samples.

b. Test methods

(1) Schedule: Lunar samples will be processed in the Biopreparation Laboratory according to the following schedule.

Day after receipt in LRL	Sample type
+2	Prime sample
+15	Pooled sample

(2) Preparation of lunar samples: The procedure for sample preparation is shown in figure 3.1.1-2. Lunar samples will be sieved through a 104μ sieve. Material less than 104μ will not be ground. Grinding procedures will reduce the particle size of the lunar material to about 25μ VMD. Preliminary grinding of lunar samples will be performed with a motor-driven sample crusher (model 150 Lemaire Instruments, Reno, Nevada). The apparatus will grind lunar samples to approximately 100μ. Final grinding of the lunar samples to approximately 25μ will be accomplished with an electric mortar grinder (Torsion Balance Company, model MG-2). An accurate record of each step will be maintained on the form presented in figure 3.1.1-3.

(a) The stainless-steel sample containers from the Vacuum Laboratory will be passed into the Class III Cabinetry of room 1-126 through the hypochlorite dunk tank.

(b) The stainless-steel sample containers will be equipped with an O-ring fitted cap secured by several bolts.

(c) The sample container will be opened and the lunar material in the sample container transferred to a sterile stainless-steel 104 μ mesh sieve.

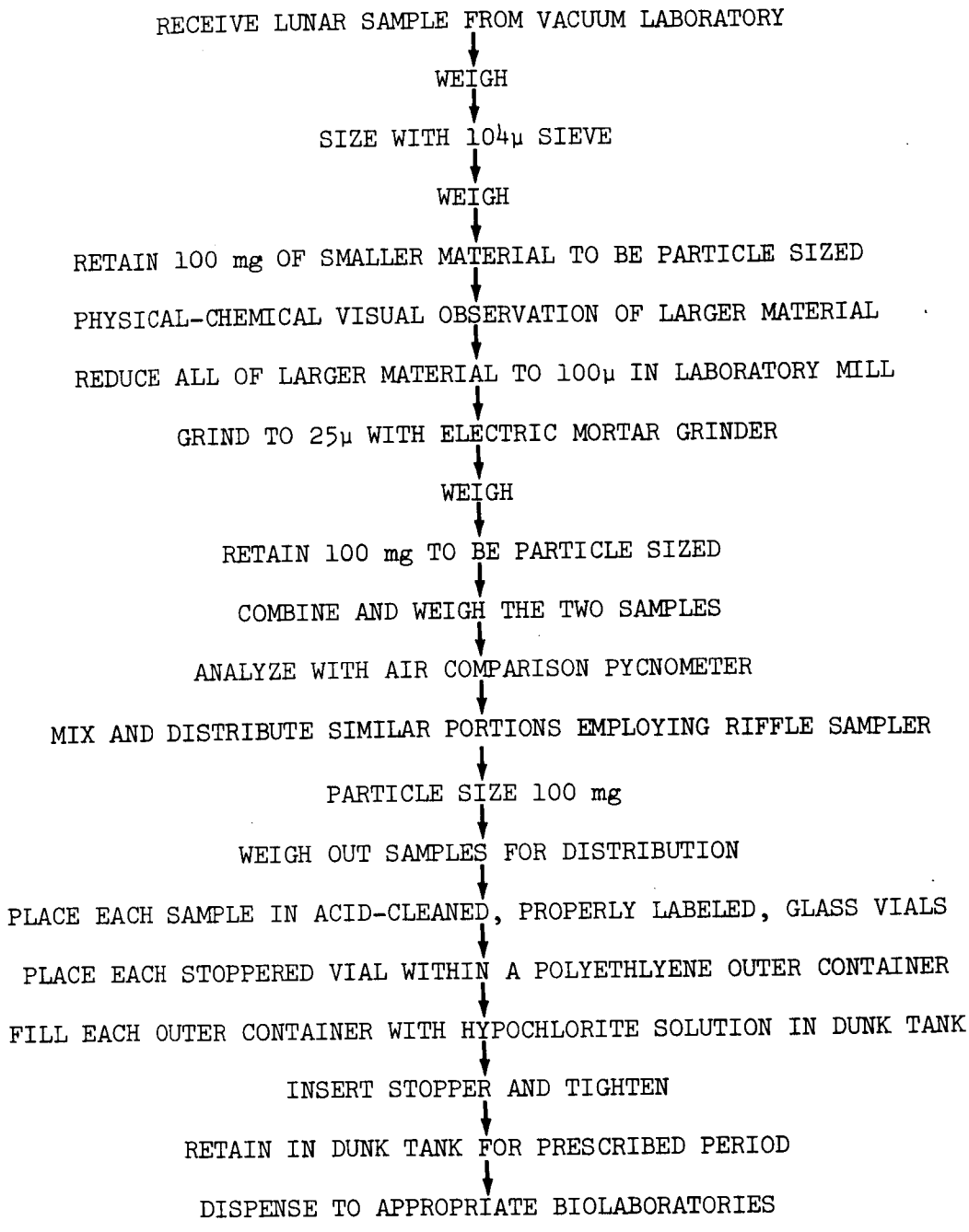


Figure 3.1.1-2.- Consolidated biopreparation procedure and particle-size distribution determination for lunar samples.

RECORD OF BIOPREPARATION ACTIVITIES

Date and Time Sample Received _____	Weight	Time started	Time completed
1. Weighing of total sample			
2. Sieving and weighing of sample			
3. Grinding in sample crusher and weighing			
4. Grinding in mortar grinder			
5. Weighing of 100-mg samples for particular sizing (sieved and ground)			
6. Mixing sieved and ground sample in cone sampler			
7. Weighing total sample			
8. Weight lost			
9. Weighing and packaging of samples to be distributed			
10. Placing of samples in sterilizer			
11. Passing out of first samples (unsterile)			
12. Passing out of the remaining samples (sterilized)			
13. Preparation of slides for particle sizing			
14. Drying time for slides			
15. Particle sizing time			
16. Calculation time			
17. Preparation of graphs			

Figure 3.1.1-3.- Form for record of biopreparation activities.

(d) The 104μ-mesh sieve will be used to separate those particles requiring pulverization. The lunar material will be spread evenly over the sieve. The sieve will be equipped with a stainless-steel collection pan and cover. The sieve will be shaken by hand until no further separation occurs.

(e) The lunar material of particle size less than 104μ will be collected in the sterile stainless-steel pan beneath the sieve. This material will not be pulverized further. It will be weighed and stored until the lunar chips have been reduced. The reduced lunar chips will then be added to this sample.

(f) The lunar material of particle size less than 104μ will be transferred to a plastic weighing boat. The material will be weighed on an analytical balance. A 100-mg aliquot will be weighed and particle sized according to procedures outlined in section 3.1.1.2b(3).

(g) Following visual physical-chemical observations, the lunar material restrained by the 104μ sieve will be transferred to a sterile plastic weighing boat. The material will be weighed on an analytical balance.

(h) The lunar sample will be poured from the weighing boat into the sterile bowl of the motor-driven sample crusher. If the weight of the lunar sample exceeds 15 grams, it will be portioned into convenient units of less than 15 grams.

(i) A sterile stainless-steel bin will be placed under the outlet of the sample crusher. Grinding of the sample will be initiated by turning the switch to the ON position.

(j) After the lunar sample has been sufficiently pulverized (about 1 minute of grinding) the pulverized sample will be transferred to a sterile mortar bowl. The mortar bowl will be attached to the electric mortar grinder.

(k) To achieve the 25μ VMD, the grinder calibration device will be adjusted to 33 mm so that the desired pressure on the pestle will be obtained.

(l) Final grinding of the lunar sample will be initiated by turning the switch to the ON position and grinding the sample for 5 minutes.

(m) The mortar bowl will be detached from the electric grinder. A 100.0-mg portion of the pulverized lunar sample will be removed for particle-size determinations as outlined in section 3.1.1.2b(3).

(n) The pulverized lunar sample will be poured from the mortar bowl into a plastic weighing boat. The ground lunar material will be weighed on an analytical balance. All of the material pulverized from one lunar sample type will be collected in a sterile pan with the material not requiring further reduction in particle size. The consolidated biopreparation procedure and particle-size distribution determinations are presented in figure 3.1.1-2.

(3) Particle-size determinations: Samples of approximately 100 mg will be sized to determine the particle-size distribution.

(a) A portion (100 mg) of the lunar sample will be weighed into a sterile beaker.

(b) Twenty milliliters of suspending fluid (200 parts of n-butyl alcohol and one part of Canada balsam) will be added to the beaker.

(c) The suspension will be stirred for 10 minutes with a stirring apparatus. During the mixing process, a small volume of the suspended lunar material will be removed with a sterile Pasteur pipette.

(d) One drop of the suspension will be transferred to each of five sterile glass slides (1 by 3 in.).

(e) The lunar suspension will be allowed to dry on each slide for a period of 2 hours.

(f) A clean, sterile glass cover slip (22 by 22 mm) will be placed over each dried lunar suspension. The corners of each cover slip will be sealed with Canada balsam.

(g) A microscope, calibrated at × 1000 magnification, equipped with a micrometer eyepiece, and attached to the Class III Cabinet, will be used to count and measure particles.

(h) Random diameters of 200 particles on each slide (total of 1000 particles) will be measured in microns and recorded.

(i) The relative volume at each of the diameter levels will be calculated by multiplying the number of particles at each size by the cube of the diameter recorded in cubic microns.

(j) The relative percent volume at each of the size levels will be calculated by dividing the accumulated volume at each level by the sum of the accumulated volumes at all levels.

(k) The relative percent volume at each size level will be plotted against the diameter (in microns) on logarithmic logic paper.

(l) The VMD will be obtained at the 50-percent intercept on the curve, whereas the slope will be expressed in terms of probits per log diameter.

(m) A record of the above calculations will be maintained in the form presented as figure 3.1.1-4.

PARTICLE SIZE DATA

OD	Frequency N	Sum N	Diameter D, microns	Percent number	Volume cubed V^3	Accumulated volume sum (ND^3)	Volume percent
1			1		1		
2			2		8		
3			3		27		
4			4		64		
5			5		125		
6			6		216		
7			7		343		
8			8		512		
9			9		729		
10			10		1 000		
11			11		1 331		
12			12		1 728		
13			13		2 197		
14			14		2 744		
15			15		3 375		
16			16		4 096		
17			17		4 913		
18			18		5 832		
19			19		6 859		
20			20		8 000		
21			21		9 261		
22			22		10 648		
23			23		12 167		
24			24		13 824		
25			25		15 625		
26			26		17 576		
27			27		19 683		
28			28		21 952		
29			29		24 389		
30			30		27 000		
31			31		29 791		
32			32		32 768		
33			33		35 937		
34			34		39 304		
35			35		42 875		
36			36		46 656		
37			37		50 653		
38			38		54 872		
39			39		59 319		
40			40		64 000		

Each OD is equal to 1.0

Figure 3.1.1.4.- Record of particle sizing calculations.

(4) Combining, sampling, and weighing of the lunar material: A Riffle sampler will be used to pool and sample the lunar material just prior to the final weighing. The prime and pooled lunar samples will be weighed and labeled according to the logistics outlined in tables 3.1.1-I and 3.1.1-II.

(a) The pulverized lunar sample and that portion not pulverized (<104μ) will be placed in a mixing bottle. The contents will be mixed by hand for 1.0 minute.

(b) The pooled lunar material will be transferred to a sterile 150-ml glass beaker and weighed on an analytical balance.

(c) An adequate amount of the mixed sample will be employed in the air comparison pycnometer for specific gravity determinations.

(d) Specific gravity calculations will be recorded on the form presented as figure 3.1.1-5.

(e) The sterile 150-ml glass beaker will be used to transfer pooled lunar material to a sterile Riffle sampler.

(f) The sample will be split into two equal parts.

(g) Additional separations will be performed in a statistical manner until aliquots of approximately the right size are obtained.

(h) The split samples will be weighed on an analytical balance (Sartorius Model 2743) which will have a weighing capacity of 100 g with a precision of ±0.1 mg. Plastic weighing boats will be used to hold lunar sample material during the weighing process.

(i) During all the operations described previously, heavy-duty aluminum foil will be used under each instrument to collect scattered particles. The foil will be replaced before weighing of different types of lunar material.

TABLE 3.1.1-I.- BIOPREPARATION OF PRIME LUNAR SAMPLE FOR APOLLO 11 MISSION

Area	Room	Condition	Quantity, g	Packaging requirement
Germ-free mice	1-127	Unsterile	13.380	100-ml vial
Germ-free mice		Sterile	13.380	
Toxicity		Unsterile	1.340	10-ml vial
Toxicity		Sterile	1.340	
			29.440	
Direct observ.	1-126	Unsterile	1.000	Standard glass vial
			1.000	
Virology	1-107			Standard glass vials
Toxicity		Sterile	4.020	
Tissue culture		Unsterile	21.460	
Tissue culture		Sterile	21.460	
Eggs		Unsterile	3.345	
Eggs		Sterile	3.345	
Mycoplasma		Unsterile	.900	
Mycoplasma		Sterile	.900	
			55.430	
Bacteriology/ mycology	1-123			Standard glass vial
Indigenous		Unsterile	2.676	
Terrestrial		Unsterile	12.488	
			15.164	
Biology total			101.034	
Chemical analysis	1-124	Sterile	2.500	Standard glass vial
Grand total			103.534	

TABLE 3.1.1-II.- BIOPREPARATION OF POOLED LUNAR SAMPLE FOR APOLLO 11 MISSION

Area	Room	Condition	Quantity, g	Packaging requirements
Germ-free mice	1-127	Unsterile	13.380	100-ml cal. serum bottle
		Sterile	2.680	30-ml cal. serum bottle
			16.060	
Direct Observ.	1-126	Unsterile	1.000	Standard glass vial
			1.000	
Virology	1-107			Standard glass vials
Tissue Culture		Unsterile	24.080	
Tissue Culture		Sterile	24.080	
Eggs		Unsterile	3.345	
Eggs		Sterile	3.345	
Mycoplasma		Unsterile	.900	
Mycoplasma		Sterile	.900	
			56.650	
Bacteriology/ mycology	1-123			Standard glass vials
Indigenous		Unsterile	2.676	
Terrestrial		Unsterile	27.206	
			29.882	
Botany	1-104			Standard glass vials
A		Unsterile	8.800	
A		Sterile	8.800	
G		Unsterile	17.600	
G		Sterile	17.600	
S-1		Unsterile	14.300	
S-2		Sterile	14.300	
S-3		Unsterile	14.300	
S-4		Sterile	14.300	
T		Unsterile	17.600	
T		Sterile	17.600	
			145.200	
Quail	1-122	Unsterile	6.690	60-ml cal. serum bottle
		Sterile	6.690	
			13.380	

TABLE 3.1.1-II.- BIOPREPARATION OF POOLED LUNAR SAMPLE FOR APOLLO 11 MISSION - Concluded

Area	Room	Condition	Quantity, g	Packaging requirements
Invert. Zool.	1-122			Standard glass vials
Protozoa		Unsterile	12.320	
Protozoa		Sterile	12.320	
Insects		Unsterile	8.580	
Insects		Sterile	8.580	
Aquatic		Unsterile	28.600	
Aquatic		Sterile	28.600	
			99.000	
Biology total			361.172	
Chemical analysis	1-124	Sterile	2.500	Standard glass vial
Grand total			363.672	

SPECIFIC GRAVITY

BECKMAN AIR COMPARISON PYCNOMETER
NASA NO. 75935

Date:_____

Operator:_____

STARTING NO._____

Sample cup wt._____

Sample	Volume (uncorr.)	Gross weight	Volume (corr.)	Net weight,g

Figure 3.1.1-5.- Data recording form for air comparison pycnometry.

(j) All samples requiring sterilization will be heated to 160° C at a partial vacuum of one-half atmosphere for 16 hours.

(5) Packaging of prepared lunar samples and removal from cabinet: The weighed lunar material will be transferred to a double-barrier container. The containers will be passed out through a dunk tank and distributed to the appropriate laboratories.

(a) The weighed lunar material contained in the plastic weighing boat will be removed from the analytical balance.

(b) Each weighed lunar sample will be poured into an acid-cleaned bottle. A sable-hair brush will be used to transfer all of the lunar material into the bottle.

(c) Bottles will be of two types: Large bottles will be single stoppered with leak-proof stoppers, and small bottles will be double stoppered with leak-proof stoppers.

(d) All bottles will be placed within leak-proof polyethylene outer containers.

(e) This outer container will be filled with hyperchlorite solution and capped.

(f) The containers will be placed in a nylon-net bag and submerged in the dunk tank.

(g) The containers will be placed in the sodium hypochlorite (5000 ppm available Cl^-) dunk tank for 30 minutes.

(6) Labeling: Dilution materials and lunar samples which enter or leave the cabinet system of room 1-126 will be labeled.

(a) Each lunar sample which arrives in the Class III Cabinetry will be labeled with a red tag as follows:

1. Type of lunar material (prime or pooled)
2. Date and time of arrival
3. Technician who received the sample

(b) The following additional information will be added to each tag as the lunar material passes through the preparation scheme.

1. Particle size
2. Date and time of pulverization

(c) Each lunar sample will be relabeled with a blue tag as it is prepared to leave the Class III Cabinetry.

1. Type of material
2. Weight of material
3. Date of preparation
4. Date lunar material left the Class III Cabinetry of room 1-126

(d) The tag will be attached to the container in which the lunar sample is stored.

(e) All dilution materials will be labeled before entrance into the Class III Cabinetry. The labels will be placed on the containers.

1. Type of material
2. Amount of material
3. Date prepared

(f) All surfaces, coming in contact with hyperchlorite and requiring labeling, will be scribed with an electric vibrator marker.

(7) Recording the operational procedures: All procedures used in weighing, pulverization, particle measurement, and packaging will be recorded.

(a) A log book will be used to record all operations occurring with each type of lunar material.

(b) One technician will record all operations performed by each technician.

c. The equipment list for materials required inside the Class III Cabinetry is delineated in table 3.1.1-III.

d. Pretest preparation of test area.

(1) All Class III Cabinetry in room 1-126 will be certified using a Freon leak-detection apparatus. The methods employed are described in appendix A of OPV7S2.8. The rubber gloves will be certified according to Fort Detrick specifications QSO-CEM-31.

(2) All Class III Cabinetry will be sterilized using paraformaldehyde. The methods used for sterilization are described in OPV7S2.

(3) All Class III Cabinetry will be supplied with a sterile nitrogen atmosphere. (Specifications for the gas will be supplied by Engineering and Operations (E&O), and certification will be made by LRL Quality Control.)

(4) All Class III Cabinetry will be sterility tested every 48 hours. One sterile, moist cotton swab will be used to sample the floor and walls of each cabinet section. Each swab will be used to streak three trypticase glucose yeast-extract agar plates. All plates will be incubated at 24° C (indigenous to cabinetry) for 5 days. The plates will be observed every 24 hours for colony formation.

TABLE 3.1.1-III.- EQUIPMENT LIST FOR MATERIALS REQUIRED INSIDE CLASS III CABINETRY

Vacuum oven	1
Vacuum pump	1
Vacuum tubing	1
In-line Millipore filters for air inlet line	3
Support stand	1
Funnels	multiple
Analytical balances	
Two each, 100 gm maximum	
One each, 500 gm maximum	
Spatulas	6
Weighing boats	multiple
Sample crusher	1
Mortar grinder	1
Metric rulers	3
Beakers	
600 ml	6
400 ml	6
50 ml	6
Graduate cylinders, 25 ml	6
104 sieve assembly	2
Microscope slides	multiple
Cover slips	multiple
n-butyl alcohol	200 ml
Canada balsam	10 ml
Applicator sticks	10
Hand mixer	1
60-mm glass Petri dish	4
Sable-hair brushes	12
Sample containers	2

TABLE 3.1.1-III.- EQUIPMENT LIST FOR MATERIALS REQUIRED INSIDE CLASS III CABINETRY - Concluded

Dunk tank container	3
Immersion oil	1 bottle
Riffle sampler	
Labels	100
Pencil	6
Writing pad	2
Microscope base	1
Slide tray	1
Stage micrometer	1
Pasteur pipettes	1 jar (40/jar)
Diamond pencil	3
Measuring spoon	3
Wrenches	6
Pycnometer	1
Aluminum foil	1 roll
Mirror	1
Trash container	1
Large jar	2
AO-light source	1
Thermometer	1
Screw driver	1
Lens paper	1 book
Sterile water	500 ml

(5) All Class III Cabinetry autoclaves and ethylene oxide gas sterilizers will be tested according to procedures outlined in the LRL Health and Safety Manual V7S3, Quarantine Control Certification.

e. Entry of equipment into cabinetry.

(1) Autoclavable materials will be sterilized as follows:

(a) Autoclavable equipment will be placed in the autoclave located in room 1-126 subsequent to cabinet sterilization.

(b) The equipment will be autoclaved at 121° C and 15 pounds of pressure for 30 minutes, employing procedures described in OMV4S9.1.1.

(c) The equipment will be removed through the inside door and passed into the cabinetry.

(2) Nonautoclavable equipment which cannot be passed through Class III Cabinetry autoclaves will be placed in open plastic bags in the carboxyclave of room 1-135A.

(a) The material will be sterilized with ethylene oxide according to the procedures outlined in OMV4S9.1.1.

(b) Each plastic bag will be sealed before removal from the carboxyclave. The bag will be transferred to room 1-126 and passed into the Class III Cabinetry through an open window.

(c) The Class III Cabinetry will subsequently be sterilized with formaldehyde employing methods described in OPV7S2.

(d) The cabinetry will be aerated for 48 hours. The bagged equipment will be opened at this time.

(e) Equipment which is not autoclavable but which can be passed through Class III Cabinetry autoclaves will be sterilized with ethylene oxide in the cabinet carboxyclave.

(f) The equipment will be removed through the inside door and passed into the cabinetry.

f. Exit of materials from cabinetry.

(1) Autoclavable materials will be sterilized as follows:

(a) Materials to be sterilized will be placed in the cabinet autoclave.

(b) The material will be autoclaved at 121° C and at 15-pounds pressure for 30 minutes using operational procedures described in Operation and Maintenance (O&M) Manual V4S9 for autoclaves in respective rooms.

(c) The material will be removed through the outside door.

(2) Nonautoclavable material will be sterilized with ethylene oxide as follows:

(a) Materials to be sterilized will be placed in the cabinet autoclave.

(b) The material to be sterilized will be exposed to ethylene oxide gas for a period of 16 hours using operational procedures described in O&M Manual V4S9 for autoclave in the respective rooms.

(c) The material will be removed through the outside door.

(3) Materials to be passed out through the dunk tank will be processed as follows:

(a) Materials will be placed in containers employing procedures described in OPV7S2.

(b) The containers will be placed in sodium hypochlorite (5000 ppm available Cl^-) dunk tank for 30 minutes.

g. Emergency plans: Overages of each type of chemical and apparatus will be available. Alternate Class III Cabinetry will be available.

(1) Approximately 100-percent overages of each of the following chemical solutions will be available within the Class III Cabinetry of room 1-126 and in the Sample Laboratory.

(a) Suspending fluid

(b) Immersion oil

(2) Surplus minor equipment available within Class III Cabinetry will be as follows:

(a) Forceps

(b) Stainless-steel collection pans

(c) Pipettes

(d) Weighing boats

(e) Glass slides

(f) Cover slips

(g) Two-ounce polypropylene bottles

(h) Six-ounce polypropylene bottles

(i) Spatulas

(j) Silicone corks

(k) Illuminator bulbs

(l) Tool kit

(m) Swabs and media

(3) Surplus major equipment available within Class III Cabinetry will be as follows:

(a) Analytical balances

(b) Light microscopes with illuminators

(4) In case of Class III Cabinetry failure within room 1-126 before receipt of lunar material, the following procedures will be incorporated:

(a) The lunar samples will be diverted from the Vacuum Laboratory to room 1-124.

(b) Without further treatment, the lunar samples will be packaged and transferred to room 1-123 for processing. Normal exit and entrance procedures will be used to transfer the lunar materials.

(c) If cabinetry failure is not extensive, the cabinetry will be repaired, resterilized, and regassed before arrival of the lunar materials.

h. Schedules

Activity	Time in reference to receipt of samples in Biopreparation cabinetry, day
Class III Cabinetry Preparation	
Sterilization	-10
Sterility testing	-8
Weighing and preparation of lunar materials	
Pulverization, grinding and combining	0
Sterilization, packaging, and transfer	+1
Particle sizing	+2

3.1.2 Direct Observation of Prime and Pooled Lunar Samples

3.1.2.1 Objectives

Prime and pooled lunar samples will be examined directly for microorganisms by employing electron microscopy, phase-contrast microscopy, white-light microscopy, and fluorescent microscopy, using appropriate staining techniques.

3.1.2.2 Protocols

a. Test design (figure 3.1.2-1)

(1) Dry samples of prime and pooled lunar material will be examined with both the light microscope (× 430, × 1000) and the stereoscope (× 7 and × 25).

(2) Aqueous extracts of prime and pooled lunar material will be concentrated and observed by phase-contrast microscopy (× 450, × 1000), and electron microscopy (× 3000 to × 80 000). The preparations will then be stained and observed by light microscopy (× 430, × 1000).

b. Test methods

The preparations of lunar samples and the microscopic observations will be performed within Class III Cabinetry in room 1-126. Samples for electron microscopy will be prepared within Class III Cabinetry, then passed out and transferred to room 189 for examination.

(1) Sample requirements

(a) One gram each of prime and pooled lunar sample.

(b) The lunar samples will be received from the biopreparation area after the pulverization and grinding procedures.

(c) Residual soil and soil extracts from tests will be stored for other programs as required.

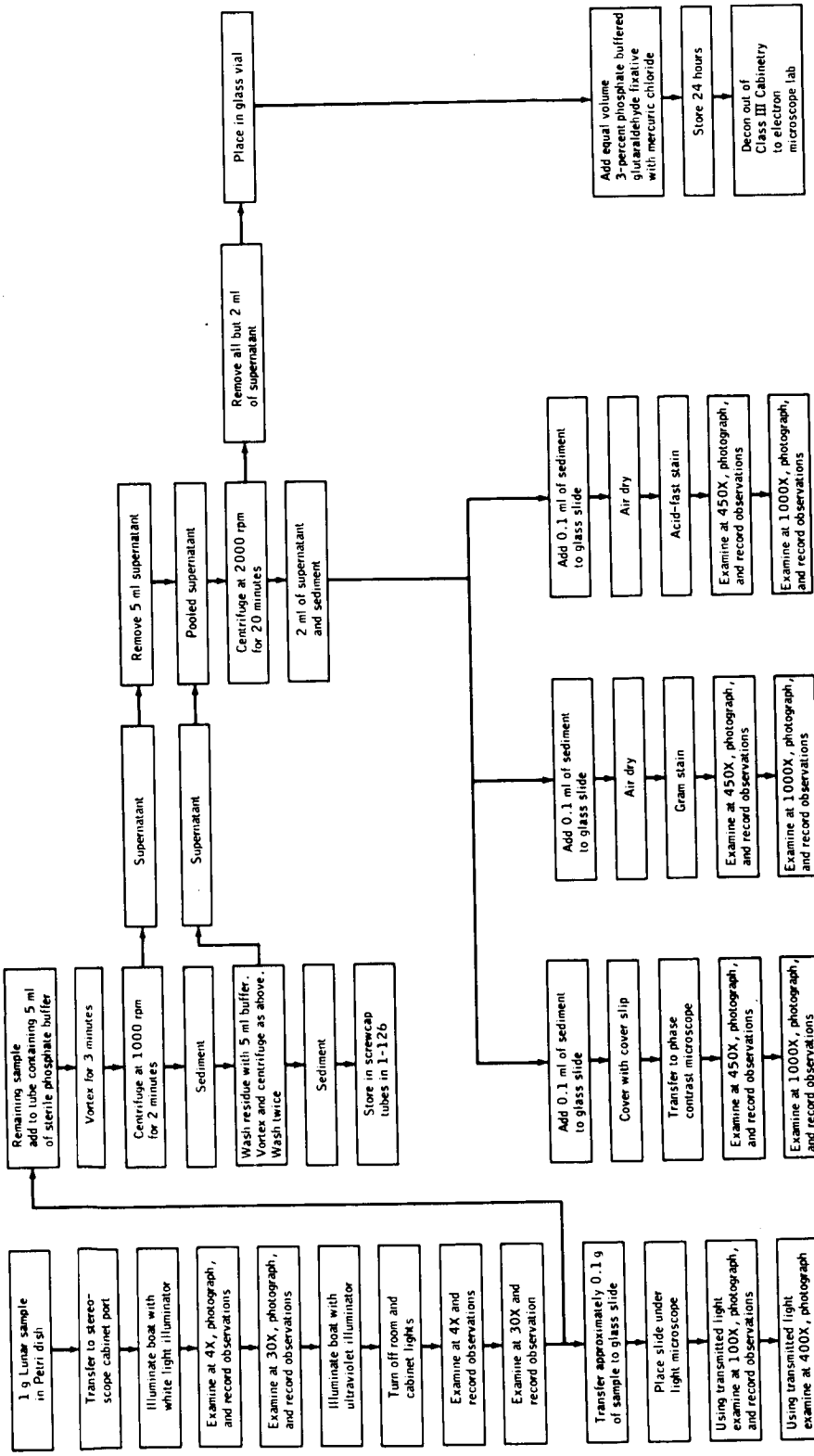


Figure 3.1.2-1.- Direct observation of prime and pooled lunar samples, room 1-126.

(2) Examination of dry lunar material for microscopic structures

(a) One gram of each type of lunar material will be placed in the bottom of a glass Petri plate using a sterile spoon-spatula. A separate Petri plate will be used for each sample.

(b) The Petri plates will be kept covered when the samples are not being observed.

(c) The bottom half of the Petri plate containing the dry lunar material will be placed under a three-dimensional stereoscope with × 7 and × 25 magnification capability.

(d) Each sample of lunar material will be illuminated with two oblique white-light illuminators and examined under the stereoscope with the aid of a needle probe.

(e) Each sample of lunar material will be scanned with two oblique ultraviolet light illuminators (λ 2537 Å) while general lighting in the room and Class III Cabinetry is turned off.

(f) All observations will be recorded.

(g) Color photoslides (35 mm) will be made from exposures of representative samples under white light and selected samples under ultraviolet light with the previous magnification.

(h) A sterile spatula will be used to transfer a small portion of each sample of lunar material to a sterile, clean glass slide.

(i) The slide will be carefully placed under a light microscope.

(j) The remaining lunar material will be extracted and concentrated.

(k) The lunar material will be examined at × 100 and × 430 magnification with transmitted light and the aid of a needle probe.

(l) All observations will be recorded.

(m) Color photoslides (35 mm) will be made from representative exposures taken at the previous magnifications and light conditions.

(3) Extraction and concentration

(a) Approximately 1 gram of the lunar sample will be placed inside a sterile, clean screwcap test tube containing approximately 5.0 ml of sterile phosphate buffer at pH 7.0 with an ionic strength of 0.0003.

(b) The mixture will be vortexed for 3 minutes.

(c) The contents of the tube will be centrifuged at 1000 rpm (190 × gravity) for 2 minutes. The supernatant will be transferred to a second screwcap test tube.

(d) The residue will be washed, vortexed, and centrifuged with 5.0-ml portions of phosphate buffer an additional two times. All 5.0-ml supernatant portions will be pooled to make a total of 15.0 ml.

(e) The residue will be stored in a screwcap test tube within the Class III Cabinetry of room 1-126.

(f) The 15.0 ml of supernatant will be centrifuged at 2000 rpm (750 × gravity) for 20 minutes. All but 2.0 ml of the supernatant will be carefully removed from the tube with a sterile pipette and transferred to two glass vials for fixation and removal from the Class III Cabinetry. This portion will be employed in the electron microscopic examination.

(g) The remaining 2-ml portion will be used for light microscopic observations in the Class III Cabinetry.

(4) Phase-contrast microscopy

(a) Portions (0.1 ml) of concentrated extract of lunar material will be pipetted to three clean sterile glass slides. (Three slides will be prepared per sample.)

(b) Sterile glass cover slips will be placed over the aqueous preparations.

(c) The slide preparations will be placed under a phase-contrast microscope and examined at × 450 and × 1000 magnification.

(d) Approximately 500 fields will be examined per slide.

(e) Color (35 mm) photoslides will be obtained from representative exposures made through the phase-contrast microscope of representative fields.

(f) All observations will be recorded.

(5) Staining

(a) Portions (0.1 ml) of the concentrated extract of lunar material will be pipetted to three clean, sterile glass slides.

(b) After the material has air dried, it will be Gram stained. An important characteristic of bacteria is their ability to take the Gram stain.

(c) The following Gram-staining procedure will be used.

1. Crystal violet will be applied to the slide for 1 minute and then washed off with water.

2. Gram's iodine will be applied to the slide for 1 minute and then washed off with water.

3. The slide will be washed with 95 percent ethanol followed by a wash with water.

4. Safranin will be applied to the slide for 30 seconds and washed off with water.

5. The slide will be blotted dry.

(d) Known control slides will be stained according to the same protocol.

(e) Portions (0.1 ml) of the concentrated extract of lunar material will be pipetted to three clean, sterile glass slides. After the material has air dried, it will be acidfast stained. An important characteristic of some bacteria and most spores is their ability to be acidfast.

(f) The following acidfast staining procedure will be used.

1. Carbol-fuchsin will be applied to the slide for 4 minutes and then washed off with water. The slide will be warmed on a hotplate during this time.

2. The slide will be washed with 3 percent acid alcohol followed by a wash with water.

3. Brilliant green will be applied to the slide for 2 minutes and then washed off with water.

4. The slide will be blotted dry.

5. The slides will be placed under the light microscope and observed at × 430 and × 1000 magnification.

6. Approximately 500 fields will be examined per slide preparation.

7. Color photoslides (35 mm) will be obtained from exposures made through the light microscope.

8. All observations will be recorded.

(g) Known control slides will be stained according to the same protocol.

(6) Electron microscopy

(a) Using a sterile pipette, approximately 13.0 ml of each prepared lunar sample extract will be transferred to two sterile, airtight, 10-ml glass vials.

(b) An amount of 3-percent glutaraldehyde fixative, sufficient to completely fill each vial, will be pipetted into the airtight glass vials (section 3.1.2.2b(3)).

(c) The contents will be mixed manually for approximately 2 minutes in order to sterilize the contents. Mixing will be repeated after 12 hours.

(d) Each vial will remain in the Class III Cabinetry for 24 hours before transfer out of the cabinet.

(e) Vials should be free of all air pockets.

(f) The vials containing the lunar material will be passed out of the Class III Cabinetry through the dunk tank containing 5000 ppm available Cl^- and using a contact time of 30 minutes.

(g) The sample vials will be transferred out of the Sample Laboratory through the airlock hypochlorite dunk tank to the electron microscope in room 189, using procedures outlined in OPV7S2.

(h) Each sample will be processed according to procedures outlined in section 3.1.2.2b(4).

(i) The equipment list for materials required inside the Class III Cabinetry is delineated in table 3.1.2-I.

TABLE 3.1.2-I.- EQUIPMENT LIST FOR MATERIALS

USED INSIDE CLASS III CABINETRY

Clinical centrifuge	1
Test tubes (16 by 125 mm)	32
Sterile phosphate buffer (5 ml/tube)	16 tubes
Pasteur pipettes (18 pipettes/tube)	2 tubes
Microscope slides	100
Cover slips	100
Spatulas	4
Glass Petri dishes (60 mm diameter)	4
Glutaraldehyde	100 ml
Mercuric chloride solution	20 ml
Glass vials	8
Stoppers	8
Plastic caps	8
Vortex mixer	1
Ultraviolet light source	4
Ultraviolet light transformer	2
Lab jack	1
Gram-stain reagents	1 set
Acidfast stain reagents	1 set
Staining rack	1
Large forceps	1
Small forceps	2
Hotplate	1
Immersion oil	1 bottle
Timer	1
Water bottles	4
Plastic wash bottles	1
Laboratory tech staining tray	1

TABLE 3.1.2-I.- EQUIPMENT LIST FOR MATERIALS

USED INSIDE CLASS III CABINETRY - Concluded

Gram-stain control cultures	1
Acidfast stain control cultures	1
Fluorescent control	1
Propipettes	2
Rubber bulbs	2
100-mm glass Petri plate with glass rod	1
Test tube rack (40 hole)	1
Vaseline	1 bottle
Applicators	10
A-O light source	2
Microscope base	2
Lens paper	2 books
uv filter for microscope	1
Cellowipes	2 boxes
Trash container	1
Container for discarded pipette	1

c. Pretest preparation of test area

(1) Class III Cabinetry (room 1-126)

(a) All Class III Cabinetry will be certified for 100-percent isolation using a Freon-leak detection apparatus. The methods used for certification are described in appendix A of OPV7S2.

(b) All Class III Cabinetry will be sterilized using paraformaldehyde. The methods used for sterilization are described in OPV7S2.

(c) All Class III Cabinetry will be sterility tested every 48 hours.

1. One sterile, moist cotton swab will be used to sample the floor and walls of each 3-foot cabinet section.

2. The swab will be used to streak one trypticase glucose yeast (TGY) extract agar plate. Each plate will be incubated at 25° C (indigenous to cabinetry) for 5 days. The plates will be observed every 24 hours for colony formation.

(d) All Class III Cabinetry will be supplied with dry sterile nitrogen (specifications for the gas will be supplied by E&O).

(e) The nitrogen gas used to meet the atmospheric requirements within the Class III Cabinetry will be certified by LRL Quality Control.

(2) Sterilization apparatus

All Class III Cabinetry autoclaves and ethylene oxide sterilizers will be tested according to procedures outlined in OPV7S2.

(3) Entry of equipment into Class III Cabinetry

(a) Autoclavable equipment will be placed in the autoclave located in room 1-126 subsequent to cabinet sterilization.

(b) The equipment will be autoclaved at 121° and 15 pounds of pressure for 30 minutes employing procedures described in OMV4S9.1.1.

(c) The equipment will be removed through the inside door and passed into the cabinetry.

(d) Nonautoclavable equipment which cannot be passed through Class III autoclaves will be placed in open plastic bags in the carboxyclave of room 1-135-A.

(e) The material will be sterilized with ethylene oxide according to the procedures outlined in OMV4S9.1.1.

(f) Each plastic bag will be sealed before removal from the carboxyclave.

(g) The bag will be transferred to room 1-126 and passed into Class III Cabinetry through an open window.

(h) The Class III Cabinetry will subsequently be sterilized with steam formaldehyde employing methods described in OPV7S2.

(i) The cabinetry will be aerated for 48 hours. The bagged equipment will be opened at this time.

(j) Equipment which is not autoclavable but can be passed through Class III Cabinetry autoclaves will be sterilized with ethylene oxide for 16 hours.

(k) The equipment will be removed through the inside door and passed into the cabinetry.

d. Emergency Plans

(1) Equipment failure

(a) Duplicate microscopes will be available within the Class III Cabinetry of room 1-126.

(b) Microscopes will also be available within Class III Cabinetry of room 1-123.

(2) Technician loss: Backup personnel will be available from the microbiology group.

(3) Cabinet failure: Alternate Class III Cabinetry in room 1-128 will be available in case of primary cabinet failure.

e. Manpower requirements and time schedules

Activity	Man-hours	Time in reference to receipt of sample in Biopreparation Cabinetry, day
Class III Cabinetry preparation:		
Sterilization	2	-8
Sterility testing	3	-6
Preparation of samples	6	+1
Observations:		
Dry mounts	3	+2
Wet mounts	4	+2
Electron microscopy	12	+3

3.1.2.3 Preparation of 3.0-Percent Phosphate-Buffered Glutaraldehyde Fixative

a. Solution A will contain 2.26 grams of monobasic sodium phosphate (NaH_2PO_4) in 100.0 ml of deionized distilled water.

b. Solution B will contain 2.52 grams of sodium hydroxide (NaOH) in 100.0 ml of deionized distilled water.

c. Solution C will contain 5.40 grams of dextrose in 100 ml of deionized distilled water.

d. Solution D will contain 1.00 gram of calcium chloride ($CaCl_2$) in 100 ml of deionized distilled water.

e. Phosphate buffer (pH 7.0 to 7.4) will contain 83.0 ml of solution A (NaH_2PO_4) and 17 ml of solution B (NaOH). A 10.0-ml aliquot of the mixture will be removed. Ten milliliters of solution C (dextrose) and 0.5 ml of solution D ($CaCl_2$) will be added to the mixture and stirred.

f. Phosphate-buffered glutaraldehyde will be prepared by adding 98.00 ml of the prepared phosphate buffer to 6.0 ml of 50 percent glutaraldehyde. One gram of mercuric chloride ($HgCl_2$) will be added to the phosphate-buffered glutaraldehyde.

g. The resulting solution will be adjusted to final pH of 7.5.

3.1.2.4 Preparation of Samples for Electron Microscopy (Figure 3.1.2-2)

a. Preparation of Epon block

(1) The material received from the Sample Laboratory will be transferred to two 15-ml centrifuge tubes. The material will be centrifuged at 35 000 rpm (100 000 × gravity) at 4° C for 1 hour.

(2) The supernatant will be transferred to a 4-dram glass vial and stored at 4° C.

(3) The pellet remaining in the centrifuge tube will be washed with 2 ml of phosphate buffer for 1 hour. Caution will be taken not to disturb the pellet. If the pellet is disrupted, it will be recentrifuged as described in step (1).

(4) The buffer will be decanted.

(5) Two milliliters of 2 percent osmic acid in phosphate buffer will be used to fix the extracted material. After 1 hour, the buffered osmic acid will be decanted.

(6) The pellet will be washed for 15 minutes in phosphate buffer. The wash will be repeated once.

(7) The pellet will be dehydrated in successive 2-ml baths of 50, 70, 95, and 100 percent ethyl alcohol.

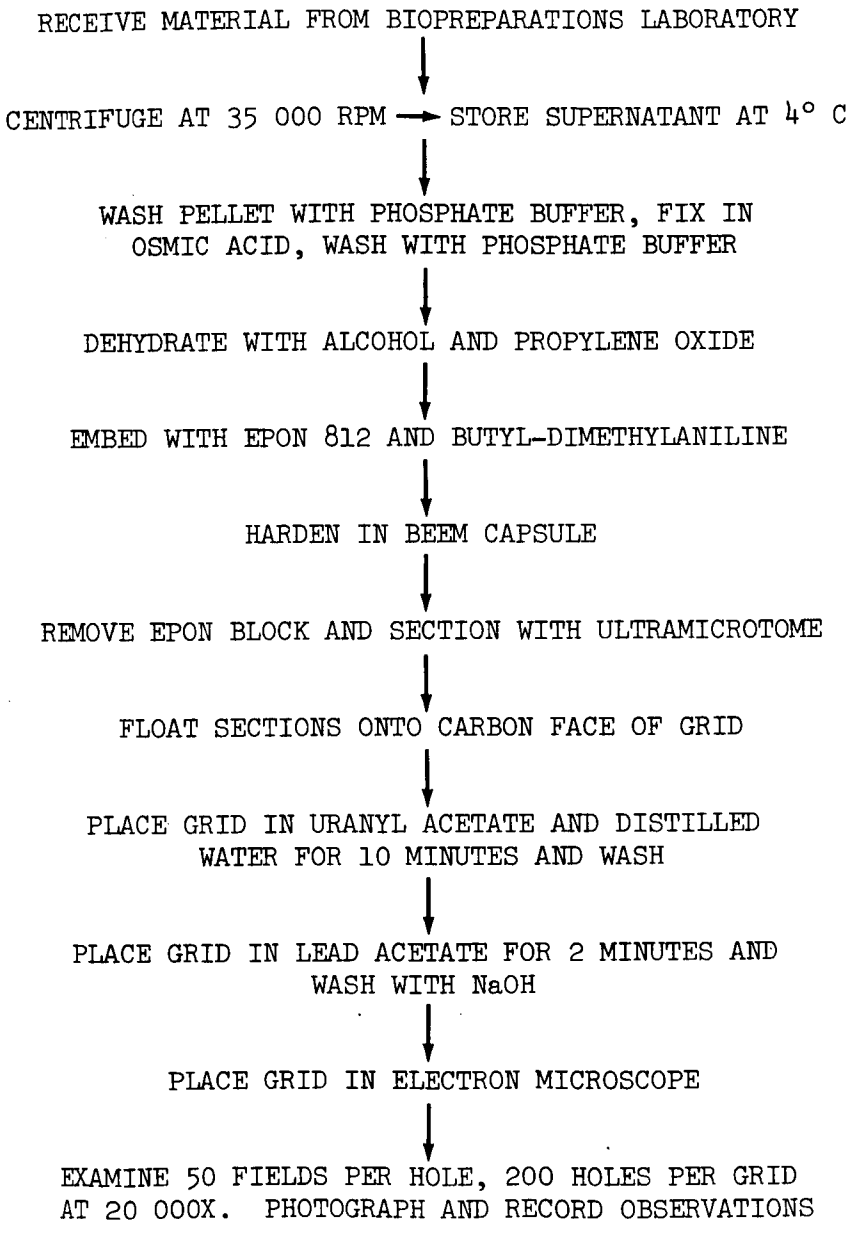


Figure 3.1.2-2.- Electron microscopic examination of prime and conventional lunar samples.

The contact period of each bath will be 20 minutes. The 100-percent ethyl alcohol bath will be repeated once. Each bath will be discarded after use.

(8) The pellet will be dehydrated in 2 ml of propylene oxide for 15 minutes. The process will be repeated once. The propylene oxide will be discarded.

(9) The pellet will be removed with a spatula and transferred to an 8-dram vial containing 2 ml of a 1:1 mixture of Epon 812 and propylene oxide with 3 percent butyl-dimethyl aniline (BDMA). The contact period will be 2 hours.

(10) An equal portion of 3-percent BDMA will be added and mixed. The contact period will be 14 hours (overnight).

(11) Fresh Epon with 3-percent BDMA will be prepared. One drop of the mixture will be added to each Beem capsule.

(12) The pellet, in approximately 1 mm^3 pieces, will be placed in each capsule and fresh Epon added until the capsules have been filled.

(13) The Epon mixture will be allowed to harden in a dry heat oven at 60° C.

(14) After the Epon has hardened, the capsules will be removed from the oven.

(15) After the capsules have cooled to room temperature, they will be sectioned or stored.

(16) The lid will be broken off each Beem capsule.

(17) Using a single-edge razor blade, the remaining capsule will be cut into thirds along the length of the capsule.

(18) The sections will be peeled backward in order to separate the Epon block from the capsule shell.

b. Cutting and mounting tissue sections

(1) Each Epon block will be secured in a collet-type holder of the Sorvall ultramicrotome so that the tissue end of the Epon block will be 3/16 inch from the front face of the holder.

(2) The collet-type holder containing the Epon block will be mounted in a trimming block.

(3) The trimming block will be fitted to the microtome table by raising the knife-stage rotation lock and sliding the block onto the table so that the small turret that projects from the table will engage the slot in the block. The lock is left loose to allow the block to rotate as the specimen is trimmed.

(4) A single-edge razor blade will be used to shape the Epon block into a truncated pyramid with sides of approximately 1 mm.

(5) The face of the block will be trimmed to a trapezoid. The sections will be made from the front face. In general, the block face will be no larger than 1 mm.

(6) The collet-type holder will be removed from the trimming block and mounted on the end of the cantilever arm. The holder is held in place by the rotation-locking-ring thread in the rear of the specimen-holder mount. This locking ring will be finger tightened and then locked using the tool provided.

(7) A diamond knife or the glass knife will be inserted into its holder.

(8) The knife holder will be inserted into the knife-stage assembly by raising the locking lever and sliding the holder into its slot from the rear.

(9) The holder will be set at the desired clearance angle referring to the scale (graduated in 2° divisions) on the right side of the holder.

(10) The holder will be locked in place by downward pressure on the locking lever. The best clearance angle is 3° to 5°.

(11) The microtome-advance system will be activated by first depressing the RESET button followed by depression of the MOTOR button. The upper thickness control will be set to assure a desired setting of 10.

(12) The OFF button will be depressed and the hand wheel rotated in a clockwise direction through at least one complete cycle and stopped with the block slightly above its midpoint in the downward cycle so that the cantilever arm will be in its forward position.

(13) The knife-advance and lateral-movement locks must be loosened and the knife brought to within 1 mm of the specimen and alined with respect to the block.

(14) Both the knife-advance and lateral-movement locks will be tightened.

(15) The fine advance-engagement screw will be loosened so that the knife advance will be in its coarse position allowing the knife to be advanced so that a small space is left between the specimen and the knife edge.

(16) The fine advance-engagement screw will be tightened to allow the forward advance to be in its fine position. The knife-stage rotation lock at the left side of the table will be unlocked by raising it against the stop.

(17) The stage will be rotated until the knife appears to be parallel to the front face of the block.

(18) The knife stage will be locked in this position by pressing down on the knife-stage rotation.

(19) The knife trough will be filled with distilled water.

(20) The ON button will be depressed. Adjustment of the tissue thickness will be made to about 600 Å or less. Adjustment of the speed will be made to about 0.35 mm/sec.

(21) Six to 10 tissue sections will be cut and floated into the knife trough.

(22) The sections will be picked up on the carbon side of a 3.2-mm grid.

(23) The grid will be blotted dry by touching its edge to the filter paper.

(24) The grid will be placed carbon-side down on a drop of 50 percent uranyl acetate in distilled water.

(25) The grid will remain in this configuration for 10 minutes.

(26) The grid will be washed by dipping in distilled water.

(27) The grid will be placed carbon-side down in a lead citrate solution for 2 minutes.

(28) The grid will be washed in successive baths of a 0.025N solution of sodium hydroxide and distilled water.

(29) The grid will be blotted dry by touching its edge to filter paper. It will then be inserted into the electron microscope.

(30) For each pellet, three grids will be examined. Within each grid, 200 holes will be surveyed. Fifty fields will be examined per hole (× 20 000).

(31) Photographs will be made of representative fields and of all fields showing evidence of organic structures.

3.2 BACTERIOLOGY AND MYCOLOGY LABORATORY

3.2.1 Crew Bacteriology and Mycology

3.2.1.1 Objectives

a. To identify possible causative agents of illnesses which may occur during the postflight quarantine period

b. To provide data on microorganisms recovered from the crewmen postflight, crew clothing, or spacecraft samples

(1) To compare the data of unknown or "suspicious" microorganisms with preflight base-line information to assist in establishing the origin as terrestrial or extraterrestrial

(2) To compare these data with information collected from microorganisms which may be isolated in the Biotest Systems to assist in establishing the origin as terrestrial or extraterrestrial

3.2.1.2 Sample Areas

3.2.1.2.1 Crew microbiology.- Eleven samples will be taken from each crewmember immediately after recovery.

a. External swabs: Two calcium alginate swabs (dampened with phosphate buffer) will be taken from each designated area. One swab will be placed in a screw-cap tube containing 10.0 ml of sterile trypticase soy broth (TSB). The second swab will be placed in a screwcap tube containing 10.0 ml of sterile veal infusion broth (VIB). The broth tubes will be maintained at 4° C during transportation to the laboratory and during dilution procedures.

(1) Scalp: An area 2 sq in. up from the hairline at the base of neck will be sampled with two swabs.

(2) External auditory canals: The right and left auditory canals will be sampled with each of two swabs. At least two revolutions will be made with each swab in each canal.

(3) Axillae: An area 1 sq in. below hair area of the left and right axillae will be sampled with the same two swabs.

(4) Umbilicus: The internal area of the umbilicus and a surrounding 2-sq in. area will be sampled with two swabs. At least two revolutions will be made with each swab.

(5) Inguinal region: A 2-inch strip from front to rear on the left and right groin areas between the legs will be sampled with each of two swabs.

(6) Toe webs: An area between the large and first toe of the right and left feet will be sampled with each of two swabs.

(7) Hands: An area of 1 sq in. on the right and on the left palms will be sampled with each of two swabs.

b. Nasal passages: Both nostrils of each crewmember will be sampled with each of two swabs. One swab will be placed in a screwcap tube containing 10.0 ml of sterile TSB. The second swab will be placed in a screwcap tube containing 10.0 ml of sterile VIB. The broth tubes will be maintained at 4° C during transportation to the laboratory and during dilution procedures.

c. Throat-mouth gargle:

(1) Each crewmember will gargle with 60.0 ml of phosphate buffer.

(2) The gargle wash will be rinsed through the oral cavity three times.

(3) The wash will be emptied into a wide-mouth bottle containing 20 ml of quadruple strength tryptose phosphate buffer.

(4) The wash containers will be maintained at 4° C during transportation to the laboratory and during dilution procedures.

d. Urine: A midstream urine sample will be taken from each crewmember; 60 ml of urine will be collected in a sterile container. The urine will be maintained at 4° C during transportation to the laboratory and during dilution procedures.

e. Feces: A stool sample from each crewmember will be obtained in a stool collection device (SCD) as near to each designated sampling time as possible. The stool samples will be stored in a hydrogen atmosphere at 4° C during transportation to the laboratory.

3.2.1.2.2 Spacecraft hardware microbiology.- Four samples will be taken from the command module hardware at the designated times. Two calcium alginate swabs (dampened with phosphate buffer) will be used to sample each designated area. One swab will be placed in a screwcap tube containing 5.0 ml of sterile TSB. The second swab will be placed in a screwcap tube containing 5.0 ml of sterile VIB. The broth tubes will be maintained at 4° C during transportation to the laboratory and during dilution procedures.

a. Floor: An area 4 sq in. on the floor of the spacecraft will be sampled with two swabs.

b. Maneuvering knob: An area 2 inches on each side of the maneuvering knob will be sampled with two swabs.

c. X-X head struts: An area of 2 sq in. on each of the two struts will be sampled with two swabs.

d. Drink gun: The total area of the length of the mouthpiece of the drink gun will be sampled with two swabs.

3.2.1.2.3 Astronaut clothing microbiology.- Three samples will be taken from each crewmember suit at the designated times. Two calcium alginate swabs (damped with phosphate buffer) will be used to sample each designated area. One swab will be placed in a screwcap tube containing 5.0 ml of sterile TSB. The second swab will be placed in a screwcap tube containing 5.0 ml of sterile VIB. The broth tubes will be maintained at 4° C during transportation to the laboratory and during dilution procedures.

a. Gloves: An area of 1 sq in. on the right glove and 1 sq in. on the left glove will be sampled with each of two swabs.

b. Shoe soles: The soles will be sampled with each of two swabs, 1 sq in. per shoe sole.

c. Urine collection device: Each suit urine collection device (UCD) will be sampled with two swabs.

3.2.1.3 Bacteriology (Figs. 3.2.1-1 to 3.2.1-5)

3.2.1.3.1 External swabs.- The scalp, external auditory canal, axillae, umbilicus, inquinal region, and toe-web samples will be incubated at 37° C until turbid, then stored at 4° C for use as contingencies dictate. The remaining samples will be processed as follows.

a. Dilution: TSB sample tubes used for aerobic identification and quantitation will be diluted serially in sterile TSB and VIB sample tubes used for anaerobic identification and quantitation will be diluted serially in sterile VIB. The sample and dilution tubes will be maintained at 4° C employing an ice bath during the dilution procedures.

(1) The sample TSB and VIB tubes will be vortexed for 5 seconds.

(2) Serial dilutions will be prepared by transferring 1.0-ml aliquots to 9.0 ml of sterile TSB or VIB.

(3) The samples will be diluted in TSB and VIB as follows (10^1 dilution represents first dilution after the sample tube).

(a) External auditory canal: TSB 10^1 to 10^4, VIB 10^1 to 10^4

(b) Hands: TSB 10^1 to 10^3, VIB 10^1 to 10^4

b. Plating: One-tenth milliliter will be transferred aseptically from each sample and each dilution TSB tube to the aerobic quantitative agar. One-tenth milliliter will be transferred aseptically from each sample and each dilution VIB tube to the anaerobic quantitative agar. The agar plates will be spread with a rod.

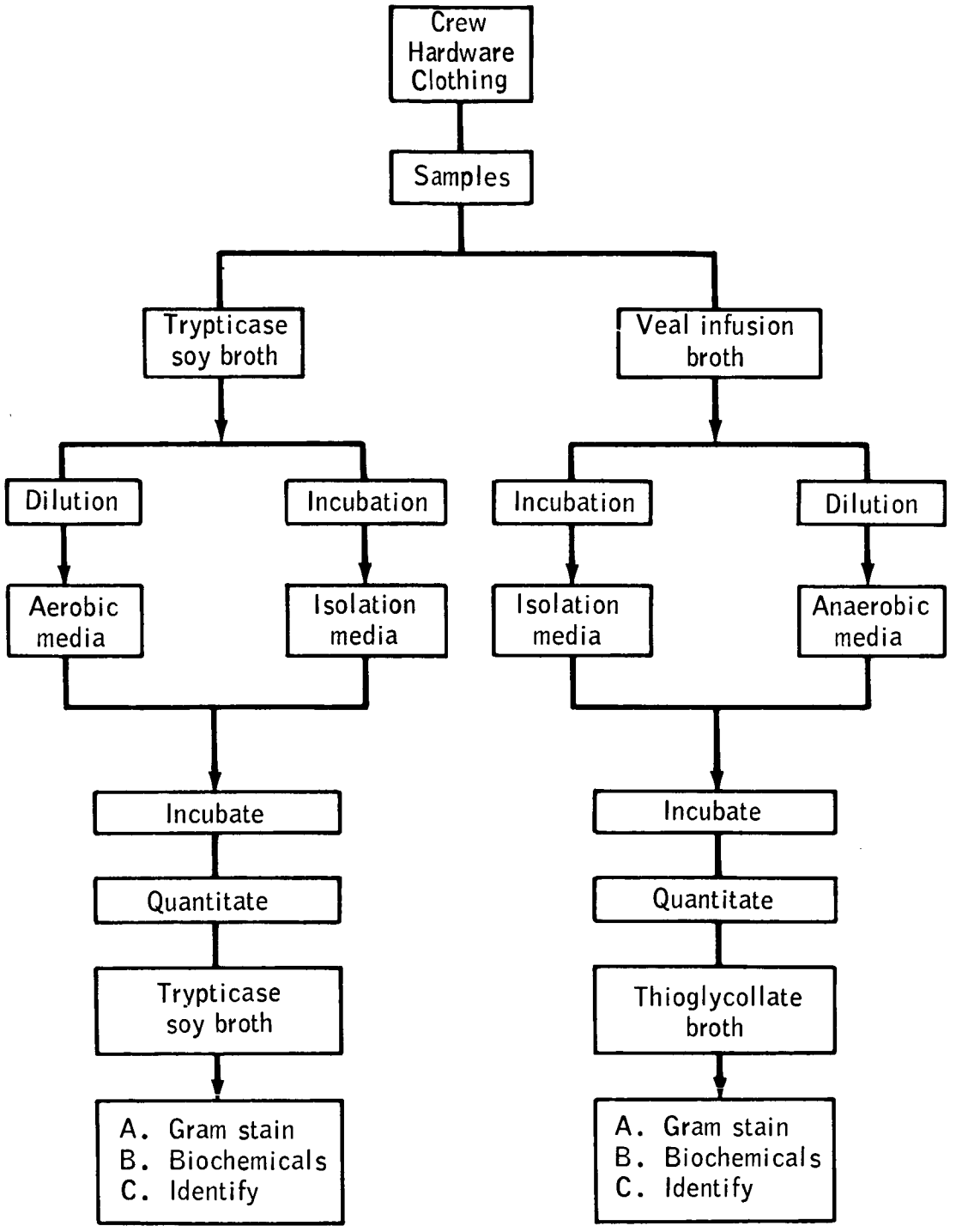


Figure 3.2.1-1.- General flow for crew hardware, and clothing samples.

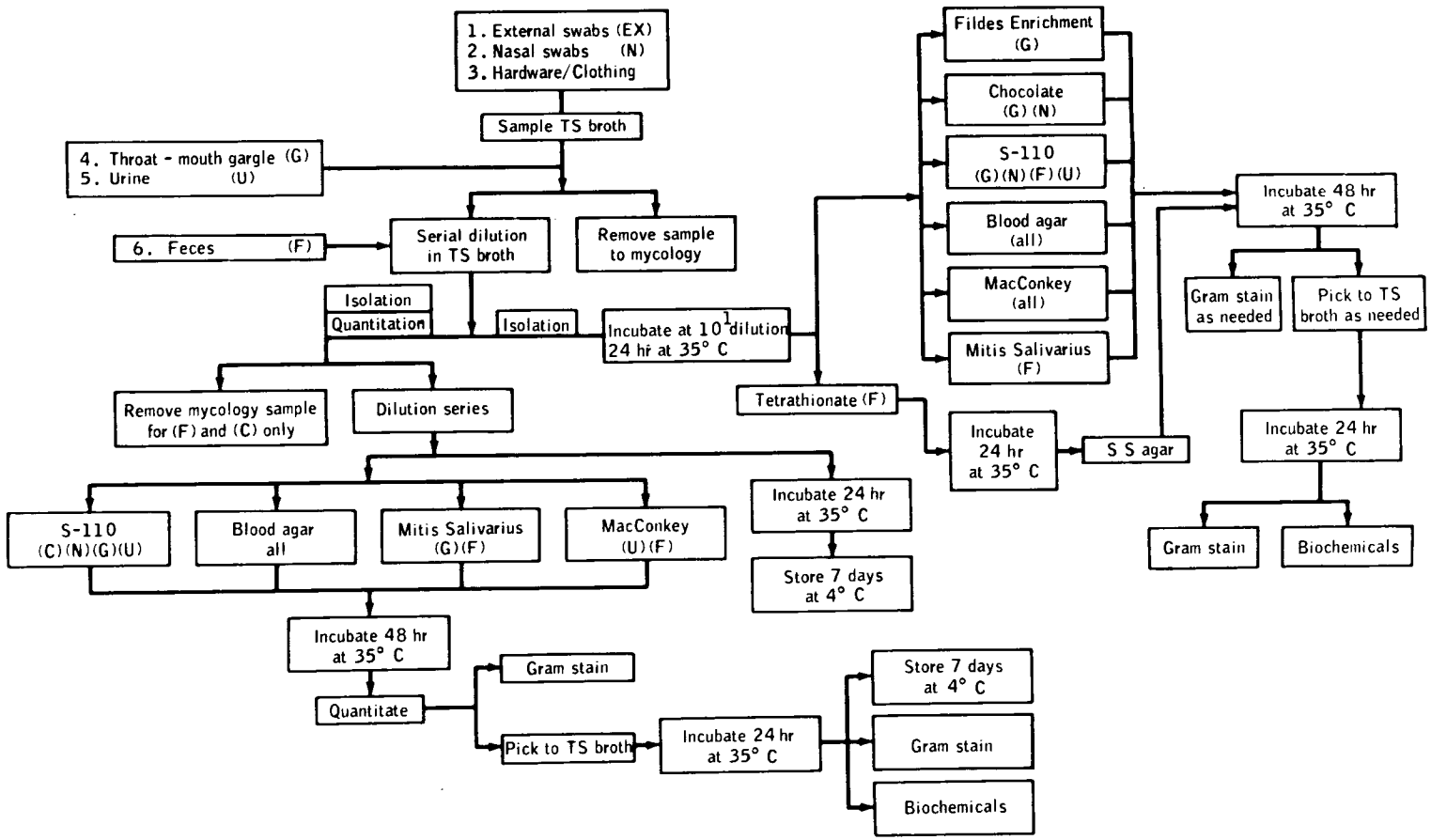


Figure 3.2.1-2.- Crew bacteriology protocol, aerobic scheme.

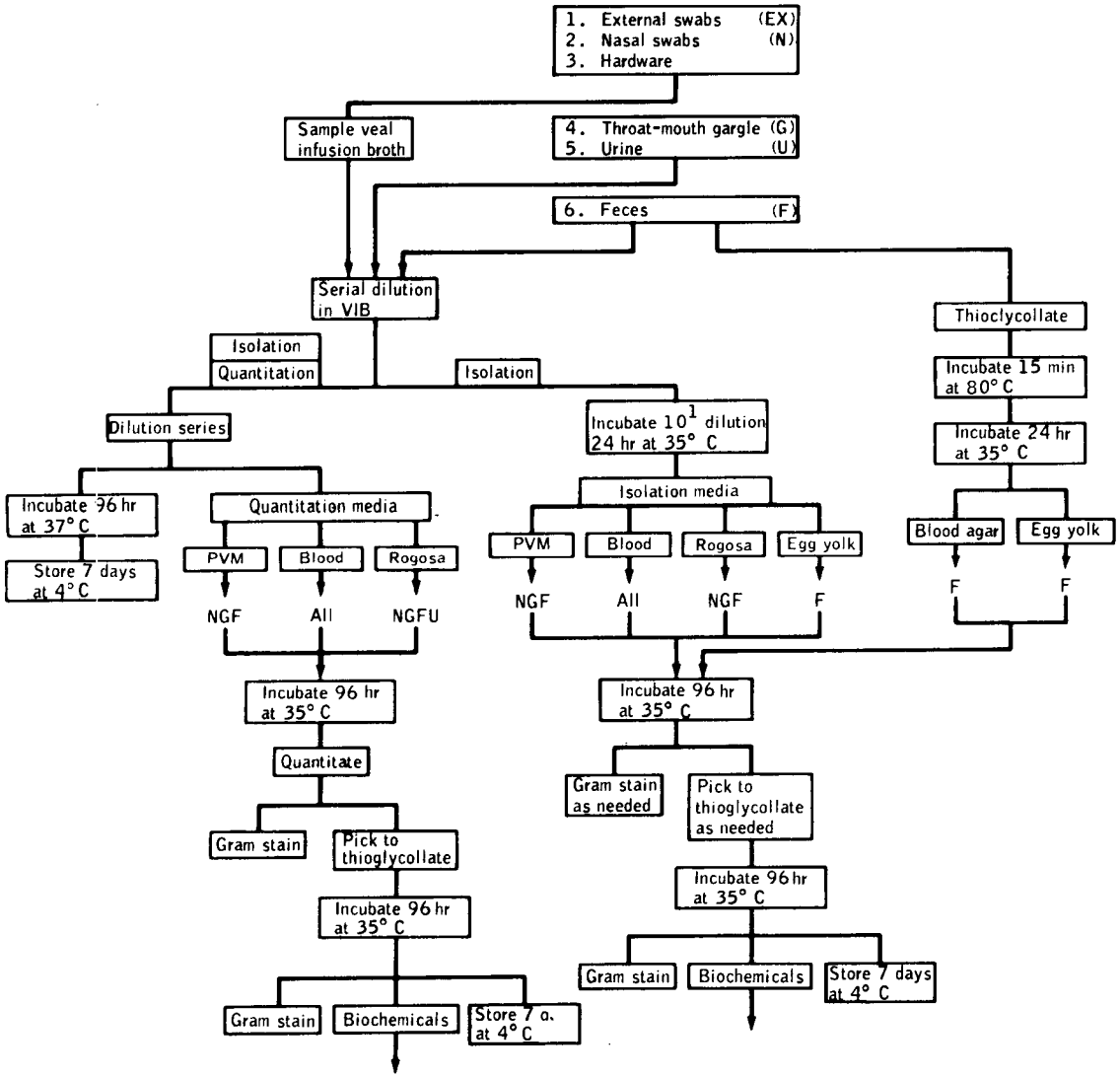


Figure 3.2.1-3.- Crew bacteriology protocol, anaerobic scheme.

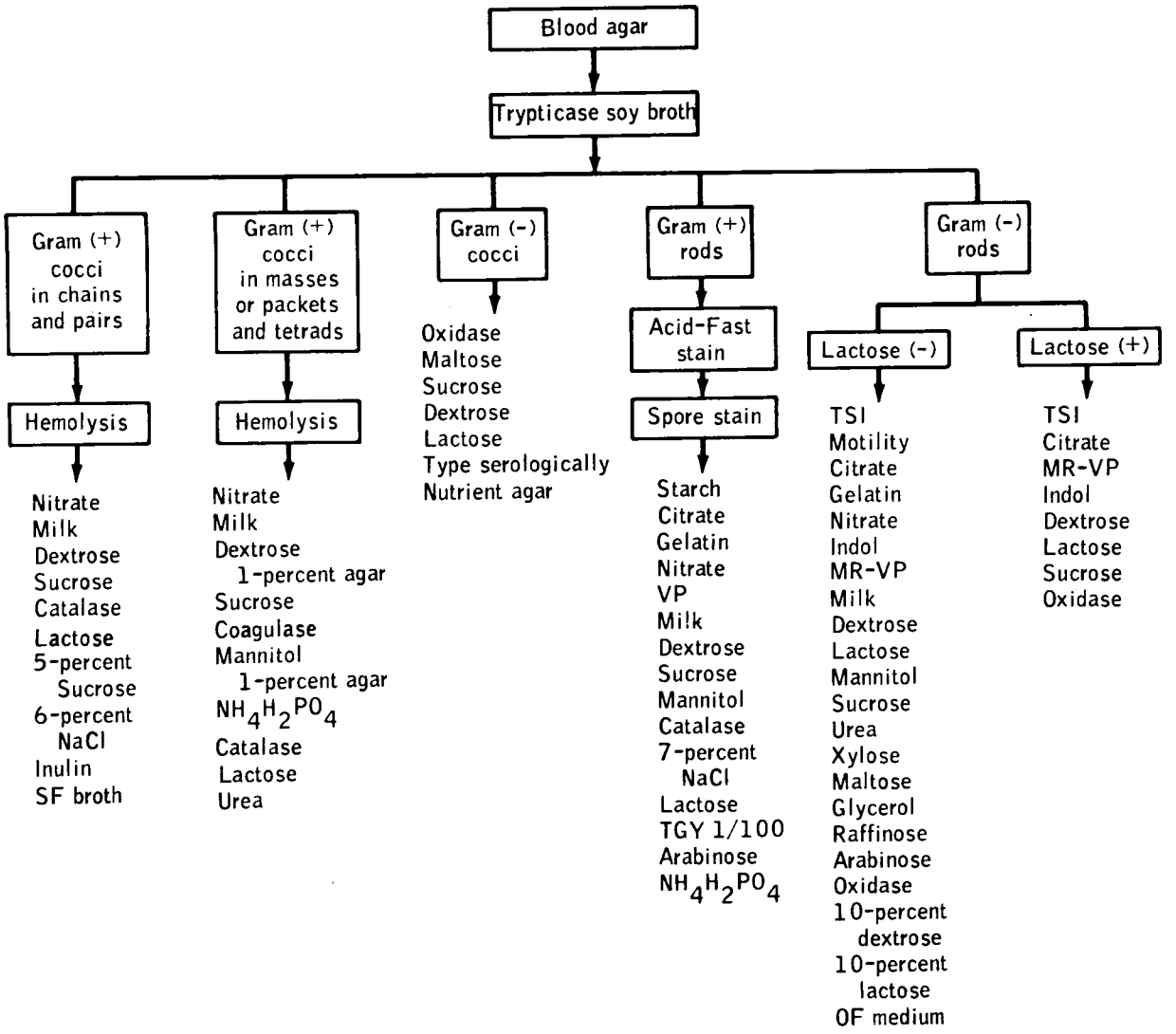


Figure 3.2.1-4.- Aerobic biochemical scheme.

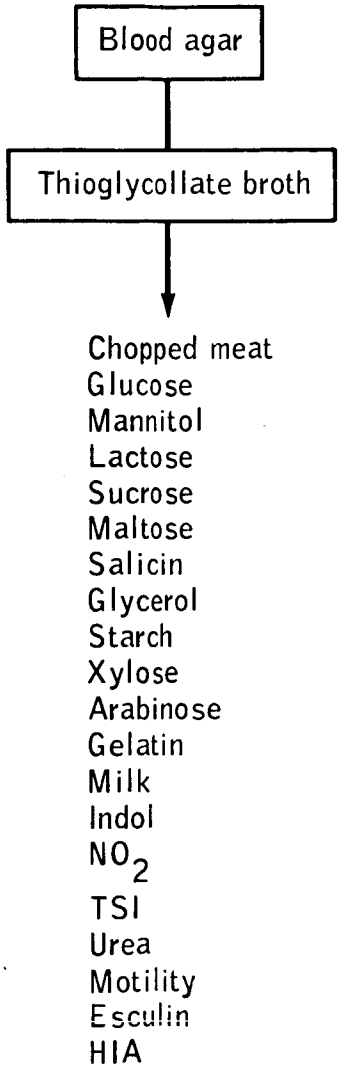


Figure 3.2.1-5.- Anaerobic biochemical scheme.

(1) The aerobic quantitative media for the external swabs include the following.

(a) Blood agar (BA)

(b) Staphylococcus-110 agar (S-110)

(2) The anaerobic quantitative media for the external swabs include blood agar with vitamin K and Hemin.

(3) Four milliliters from each TSB sample tube will be transferred aseptically to a labeled sterile screwcap tube for mycobiological analysis.

c. Incubation and quantitation:

(1) The aerobic quantitative media will be incubated at 35° C for 48 hours.

(2) The anaerobic quantitative media will be incubated at 35° C for 96 hours under an atmosphere of hydrogen gas.

(3) Colony counts will be performed on all quantitative media after incubation.

d. Isolation streaks:

(1) After mycological samples have been removed from the TSB sample tubes, the TSB and VIB sample tubes will be incubated for 24 hours at 35° C. In addition, all TSB and VIB dilution tubes will be incubated for 24 hours at 35° C and stored at 4° C for 7 days.

(2) After incubation, a loop will be used to transfer culture from each sample tube to the isolation media. An isolation streak will be made on each medium.

(3) The isolation media used for the external samples from the crew include the following.

(a) Blood agar

(b) Staphylococcus-110 agar

(c) MacConkey agar (MAC)

(d) Blood agar with vitamin K and Hemin (anaerobic)

(4) The streaked isolation media will be incubated for 48 hours or 96 hours at 35° C under the appropriate atmosphere.

3.2.1.3.2 Nasal passages.- Samples from the nasal passages will be processed as follows.

a. Dilution: All TSB sample tubes used for aerobic identification and quantitation will be diluted serially in sterile TSB. All VIB sample tubes used for anaerobic identification and quantitation will be diluted serially in sterile VIB. The sample and dilution tubes will be maintained at 4° C, employing an ice bath during the dilution procedures.

(1) The sample TSB and VIB tubes will be vortexed for 5 seconds.

(2) Serial dilutions will be prepared by transferring 1.0-ml aliquots to 9.0 ml of sterile TSB or VIB.

(3) The nasal samples will be diluted in TSB and VIB as follows (the 10^1 dilution represents the first dilution after the sample tube.

(a) TSB: 10^1 to 10^4

(b) VIB: 10^1 to 10^4

b. Plating: One-tenth milliliter will be transferred aseptically from each sample and each dilution TSB tube to the aerobic quantitative agar. One-tenth milliliter will be transferred aseptically from each sample and each dilution VIB tube to the anaerobic quantitative agar. The agar plates will be spread with a glass rod.

(1) The aerobic quantitative media for the nasal passage swabs include the following.

(a) Blood agar

(b) Staphylococcus-110 agar

(2) The anaerobic quantitative media for the nasal passage swabs include the following.

(a) Blood agar with vitamin K and Hemin

(b) Paromomycin-Vancomycin-Menadione agar (PVM)

(c) Rogosa agar (Rogosa)

(3) Four milliliters from each TSB sample tube will be transferred aseptically to a labeled sterile screwcap tube for mycobiological analysis.

c. Incubation and quantitation:

(1) The aerobic quantitative media will be incubated at 35° C for 48 hours.

(2) The anaerobic quantitative media will be incubated at 35° C for 96 hours under an atmosphere of hydrogen gas.

(3) Colony counts will be performed on all quantitative media after incubation.

d. Isolation streaks:

(1) After mycological samples have been removed from the TSB sample tubes, the sample TSB and VIB tubes will be incubated for 24 hours at 35° C. In addition, all TSB and VIB dilution tubes will be incubated for 24 hours at 35° C and stored at 4° C for 7 days.

(2) After incubation, a loop will be used to transfer cultures from each sample tube to the isolation media. An isolation streak will be made on each medium.

(3) The isolation media used for the nasal passage samples include the following.

(a) Blood agar

(b) Staphylococcus-110 agar

(c) MacConkey agar

(d) Chocolate agar (CHOC)

(e) Blood agar with vitamin K and Hemin (anaerobic)

(f) Paromomycin-Vancomycin-Menadione agar (anaerobic)

(g) Rogosa agar (anaerobic)

(4) The streaked isolation media will be incubated for 48 or 96 hours at 35° C under the appropriate atmosphere. The media (chocolate agar) incubated under CO_2 will be placed in an incubator with a CO_2 concentration of 8 to 10 percent.

3.2.1.3.3 Throat-mouth gargle.- Samples from the throat-mouth gargle will be processed as follows.

a. Dilution: All throat-mouth gargle samples will be diluted in sterile TSB or VIB for aerobic and anaerobic quantitation. The sample and the dilution tubes will be maintained at 4° C, employing an ice bath during the dilution procedure.

(1) The throat-mouth gargle sample will be swirled gently.

(2) Serial dilutions will be prepared by transferring 1.0-ml aliquots to 9.0 ml of sterile TSB or VIB.

(3) The throat-mouth gargle samples will be diluted in TSB or VIB as follows (the 10^1 dilution represents the first dilution after the sample bottle).

(a) TSB: 10^1 to 10^5

(b) VIB: 10^1 to 10^5

b. Plating: One-tenth milliliter will be transferred aseptically from each sample bottle and each dilution TSB tube to the aerobic quantitative agar. One-tenth milliliter will be transferred aseptically from each

sample bottle and dilution VIB tube to the anaerobic quantitative agar. The agar plates will be spread with a glass rod.

(1) The aerobic quantitative media for the throat-mouth gargle sample include the following.

(a) Blood agar

(b) Staphylococcus-110 agar

(c) Mitis Salivarius agar (MS)

(2) The anaerobic quantitative media for the throat-mouth gargle sample include the following.

(a) Blood agar with vitamin K and Hemin

(b) Paromomycin-Vancomycin-Menadione agar

(c) Rogosa agar

(3) Four milliliters from each sample bottle and all TSB dilution tubes will be transferred aseptically to individually labeled sterile screwcap tubes for mycobiological analysis.

c. Incubation and quantitation:

(1) The aerobic quantitative media will be incubated at 35° C for 48 hours.

(2) The anaerobic quantitative media will be incubated at 35° C for 96 hours under an atmosphere of hydrogen gas.

(3) Colony counts will be performed on all quantitative media after incubation.

d. Isolation streaks:

(1) After mycological samples have been removed from the sample bottles, the sample bottles will be incubated for 24 hours at 35° C. In addition, all TSB and VIB dilution tubes will be incubated for 24 hours at 35° C and stored at 4° C for 7 days.

(2) After incubation, a loop will be used to transfer culture from each sample bottle to the isolation media. An isolation streak will be made on each medium.

(3) The isolation media used for the throat-mouth gargle samples include the following.

(a) Blood agar

(b) Staphylococcus-110 agar

(c) MacConkey agar

(d) Chocolate agar

(e) Fildes enrichment agar (FEA)

(f) Blood agar with vitamin K and Hemin (anaerobic)

(g) Paromomycin-Vanocomycin-Menadione agar (anaerobic)

(h) Rogosa agar (anaerobic)

(4) The streaked isolation media will be incubated for 48 to 96 hours at 35° C under the appropriate atmosphere. The media (chocolate agar) incubated under CO_2 will be placed in an incubator that has a CO_2 concentration of 8 to 10 percent.

3.2.1.3.4 Urine.- Samples of the urine will be processed as follows.

a. Dilution: All urine samples used for aerobic identification and quantitation will be diluted serially in sterile TSB. All VIB urine samples used for anaerobic identification and quantitation will be diluted serially in sterile VIB. The urine samples and dilution tubes will be maintained at 4° C, employing an ice bath during the dilution procedures.

(1) The urine sample containers will be swirled gently.

(2) Serial dilutions will be prepared by transferring 1.0-ml aliquots to 9.0 ml of sterile TSB or VIB.

(3) The urine samples will be diluted in TSB and VIB as follows (the 10^1 dilution represents the first dilution after the sample tube).

(a) TSB: 10^1 to 10_2

(b) VIB: 10^1 to 10_2

b. Plating: One-tenth milliliter will be transferred aseptically from each urine sample and dilution TSB tube to the aerobic quantitative agar. One-tenth milliliter will be transferred aseptically from each urine sample and dilution VIB tube to the anaerobic quantitative agar. The agar plates will be spread with a glass rod.

(1) The aerobic quantitative media for the urine samples include the following.

(a) Blood agar

(b) Staphylococcus-110 agar

(c) MacConkey agar

(2) The anaerobic quantitative media for the urine samples include the following.

(a) Blood agar with vitamin K and Hemin

(b) Rogosa agar

(3) Four milliliters from each sample bottle will be transferred aseptically to a sterile screwcap tube for mycological analysis.

c. Incubation and quantitation:

(1) The aerobic quantitative media will be incubated at 35° C for 48 hours.

(2) The anaerobic quantitative media will be incubated at 35° C for 96 hours under an atmosphere of hydrogen gas.

(3) Colony counts will be performed on all quantitative media after incubation.

d. Isolation streaks:

(1) After mycological samples have been removed from the urine samples, the urine samples will be incubated for 24 hours at 35° C. In addition, all TSB and VIB dilution tubes will be incubated for 24 hours at 35° C and stored at 4° C for 7 days.

(2) After incubation, a loop will be used to transfer cultures from each urine sample to the isolation media. An isolation streak will be made on each medium.

(3) The isolation media used for the urine samples include the following.

(a) Blood agar

(b) Staphylococcus-110 agar

(c) MacConkey agar

(d) Blood agar with vitamin K and Hemin (anaerobic)

(e) Rogosa agar (anaerobic)

(4) The streaked isolation media will be incubated for 48 or 96 hours at 35° C under the appropriate atmosphere.

3.2.1.3.5 Feces: Samples of feces will be processed as follows.

a. Dilution: All stool samples used for aerobic identification and quantitation will be diluted serially in sterile TSB. All stool samples used for anaerobic identification and quantitation will be diluted serially in sterile VIB. The dilution tubes will be maintained at 4° C, employing an ice bath during the dilution procedures. A formalin-ether preparation of each stool sample will be performed for ova, cysts, and parasites.

(1) One-tenth gram from the center of the stool sample will be weighed onto inert weighing paper and transferred to 9.9 ml of sterile TSB.

(2) One-tenth gram from the center of the stool sample will be weighed onto inert weighing paper and transferred to 9.9 ml of sterile VIB.

(3) The TSB and VIB tubes containing the weighed stool samples will be vortexed for 30 seconds.

(4) Serial dilutions will be prepared by transferring 1.0-ml aliquots to 9.0 ml of sterile TSB or VIB.

(5) The samples will be diluted in TSB or VIB as follows.

(a) TSB: 10^1 to 10^6

(b) VIB: 10^1 to 10^8

b. Plating: One-tenth milliliter will be transferred aseptically from each dilution TSB tube to the aerobic quantitative agar media and tetrathionate broth (TB). One-tenth milliliter will be transferred aseptically from the 10^3 to 10^8 dilution VIB tubes to the anaerobic quantitative agar. The agar will be spread with a glass rod.

(1) The aerobic quantitative media for the stool samples include the following.

(a) Blood agar

(b) MacConkey agar

(c) Mitis Salivarius agar

(d) Tetrathionate broth

(2) The anaerobic quantitative media for the stool samples include the following.

(a) Blood agar with vitamin K and Hemin

(b) Paromomycin-Vancomycin-Menadione agar

(c) Rogosa agar

(3) Four milliliters from each TSB sample tube will be transferred aseptically to a labeled sterile screwcap tube for mycobiological analysis.

c. Incubation and quantitation:

(1) The aerobic quantitative media will be incubated at 35° C for 48 hours.

(2) The anaerobic quantitive media will be incubated at 35° C for 96 hours under an atmosphere of hydrogen gas.

(3) The tetrathionate broth will be incubated for 24 hours at 35° C.

(4) Colony counts will be performed on all quantitative media after incubation.

d. Isolation streaks:

(1) After mycological samples have been removed from the 10^1 TSB dilution tubes, the 10^1 TSB and 10^1 VIB dilution tubes will be incubated for 24 hours at 35° C. In addition, all TSB and VIB dilution tubes will be incubated for 24 hours at 35° C and stored at 4° C for 7 days.

(2) After incubation, a loop will be used to transfer culture from each sample tube to the isolation media. One loopfull of tetrathionate culture will be used to inoculate the Salmonella-Shigella (S S) agar. An isolation streak will be made on each medium.

(3) The isolation media used for the stool samples include the following.

(a) Blood agar

(b) MacConkey agar

(c) Mitis Salivarius agar

(d) Salmonella-Shigella agar

(e) Blood agar with vitamin K and Hemin (anaerobic)

(f) Paromomycin-Vancomycin-Menadione agar (anaerobic)

(g) Egg yolk agar (EYA) (anaerobic)

(h) Rogosa agar (anaerobic)

(4) The streaked isolation media will be incubated for 48 or 96 hours at 35° C under the appropriate atmosphere.

3.2.1.3.6 Hardware and clothing.- The spacecraft floor, maneuvering knob, X-X head struts, drink gun, urine collection device, and the astronaut gloves and shoe sole samples will be tested as follows.

a. Dilution: All TSB sample tubes used for aerobic identification and quantitation will be diluted serially in sterile VIB. The sample and dilution tubes will be maintained at 4° C, employing an ice bath during the dilution procedures.

(1) The sample TSB and VIB tubes will be vortexed for 5 seconds.

(2) Serial dilutions will be prepared by transferring 1.0-ml aliquots to 9.0 ml of sterile TSB or VIB.

(3) The preflight samples will be diluted in TSB and VIB as follows (the 10^1 dilution represents the first dilution after the sample tube).

(a) Floor: TSB 10^1 to 10^2; VIB 10^1 to 10^2

(b) Maneuvering knob: TSB 10^1 to 10^2; VIB 10^1 to 10^2

(c) Drink gun: TSB 10^1 to 10^2; VIB 10^1 to 10^2

(d) X-X head struts: TSB 10^1 to 10^2; VIB 10^1 to 10^2

(e) Urine collection device: TSB 10^1 to 10^2; VIB 10^1 to 10^2

(f) Gloves: TSB 10^1 to 10^2; VIB 10^1 to 10^2

(g) Shoe soles: TSB 10^1 to 10^2; VIB 10^1 to 10^2

(4) The postflight samples will be diluted an additional two logs in TSB and VIB.

b. Plating: One-tenth milliliter will be transferred aseptically from each sample and each dilution TSB tube to the aerobic quantitative agar. One-tenth milliliter will be transferred aseptically from each sample and dilution VIB tube to the anaerobic quantitative agar. The agar plates will be spread with a glass rod.

(1) Blood agar is the aerobic quantitative medium that is used for hardware and clothing swabs.

(2) Blood agar with vitamin K and Hemin is the anaerobic quantitative medium that is used for hardware and clothing swabs.

(3) Four milliliters from each TSB sample tube will be transferred aseptically to a labeled sterile screwcap tube for mycobiological analysis.

c. Incubation and quantitation:

(1) The aerobic quantitative medium will be incubated at 35° C for 48 hours.

(2) The anaerobic quantitative medium will be incubated at 35° C for 96 hours under an atmosphere of hydrogen gas.

(3) Colony counts will be performed on all quantitative media after incubation.

d. Isolation streaks:

(1) After mycological samples have been removed from the TSB sample tubes, the TSB and VIB sample tubes will be incubated for 24 hours at 35° C.

In addition, all TSB and VIB dilution tubes will be incubated for 24 hours at 35° C and stored at 4° C for 7 days.

(2) After incubation, a loop will be used to transfer cultures from each sample tube to the isolation media. An isolation streak will be made on each medium.

(3) The isolation media used for the hardware and clothing samples include the following.

(a) Blood agar

(b) MacConkey agar

(c) Blood agar with vitamin K and Hemin (anaerobic)

(4) The streaked isolation media will be incubated for 48 or 96 hours at 35° C under the appropriate atmosphere.

3.2.1.3.7 Isolation and identification procecures.-

a. Isolation:

(1) After quantitation, isolated colonies from each aerobic plate (quantitative and isolation media) will be transferred to sterile TSB. All tubes will be identified properly and incubated at 35° C until turbid. The TSB pure cultures will be used for staining procedures, inoculation of biochemical media, and for storage at 4° C.

(2) After quantitation, isolated colonies from each anaerobic plate (quantitative and isolation media) will be transferred to sterile thioglycollate broth (THIO). All tubes will be properly identified and incubated at 35° C until turbid. The THIO pure cultures will be used for staining procedures, inoculation of biochemical media, and for storage at 4° C.

(3) The isolation streak is employed to culture organisms which are too few to be isolated on the quantitative media. Only those organisms which

were not isolated on the quantitative media will be identified. These organisms will be quantitated as <100 organisms/ml of sample. The isolation media on which the organism was cultured will be recorded.

b. Identification:

(1) The pure cultures of each isolated colony (TSB or THIO) will be used to make Gram stains, spore stains, and acid-fast stains.

(2) These same cultures will be also used to inoculate biochemical media as outlined in figures 3.2.1-6 to 3.2.1-12.

3.2.1.4 Mycology

3.2.1.4.1 Throat-mouth gargle.-

a. One-tenth-milliliter aliquots will be removed from the throat-mouth gargle sample bottles and from the 10^1, 10^2, and 10^3 TSB dilution tubes and transferred to each of the following quantitative media.

(1) Corn meal-malt extract agar (CMMY) (containing antibiotics)

(2) Sabourauds dextrose agar (SDA) (containing antibiotics)

(3) Czapek Dox agar (CD)

b. The plates will be spread with a glass rod and incubated at 25° C for 120 hours.

c. Four milliliters of the throat-mouth gargle samples will each be transferred aseptically to a sterile centrifuge tube. The sample will be centrifuged at 2500 rpm for 15 minutes.

d. The supernate will be poured into 10-ml yeast-malt broth (YM) containing antibiotics, and a swab will be used to sample the bottom of the centrifuge tube.

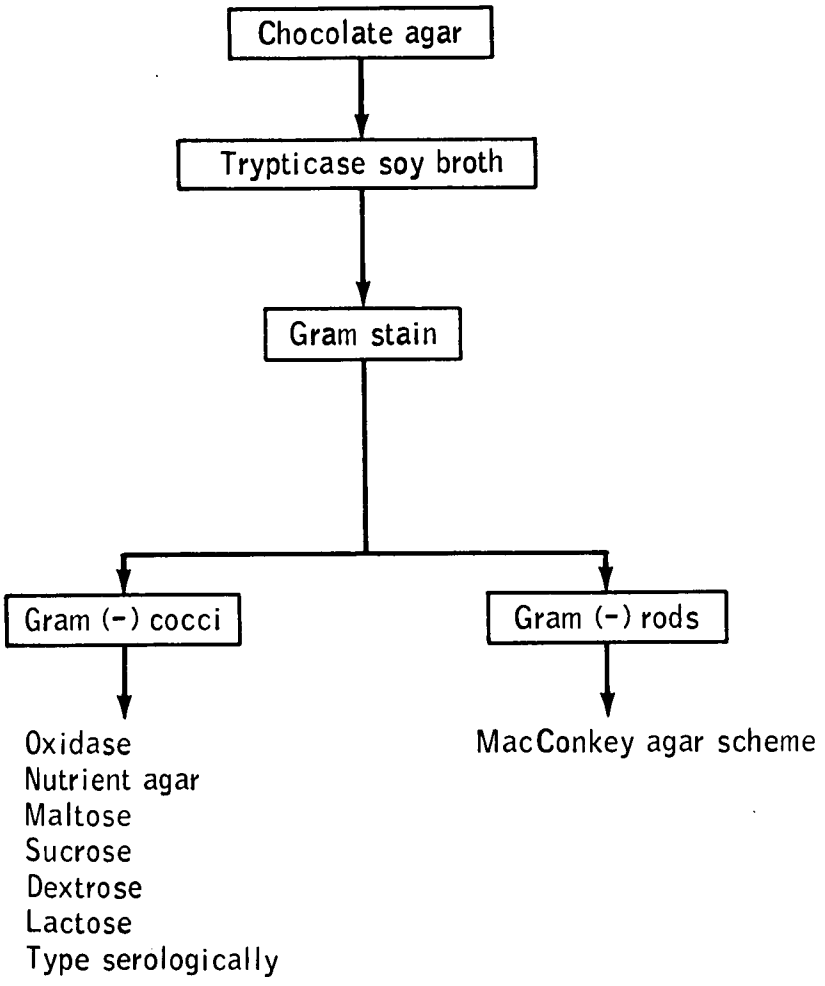


Figure 3.2.1-6.- Procedure for chocolate agar.

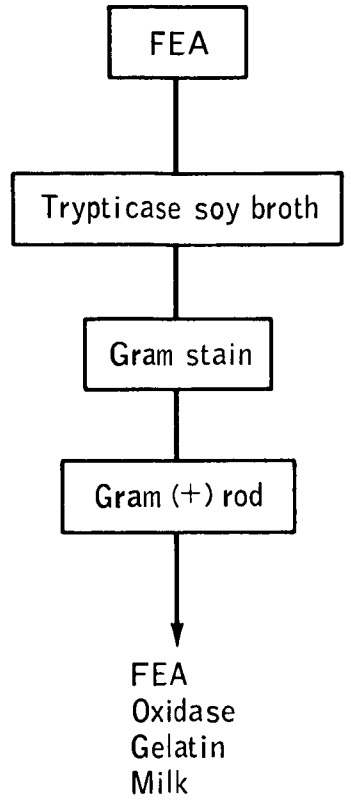


Figure 3.2.1-7.- Fildes enrichment agar scheme.

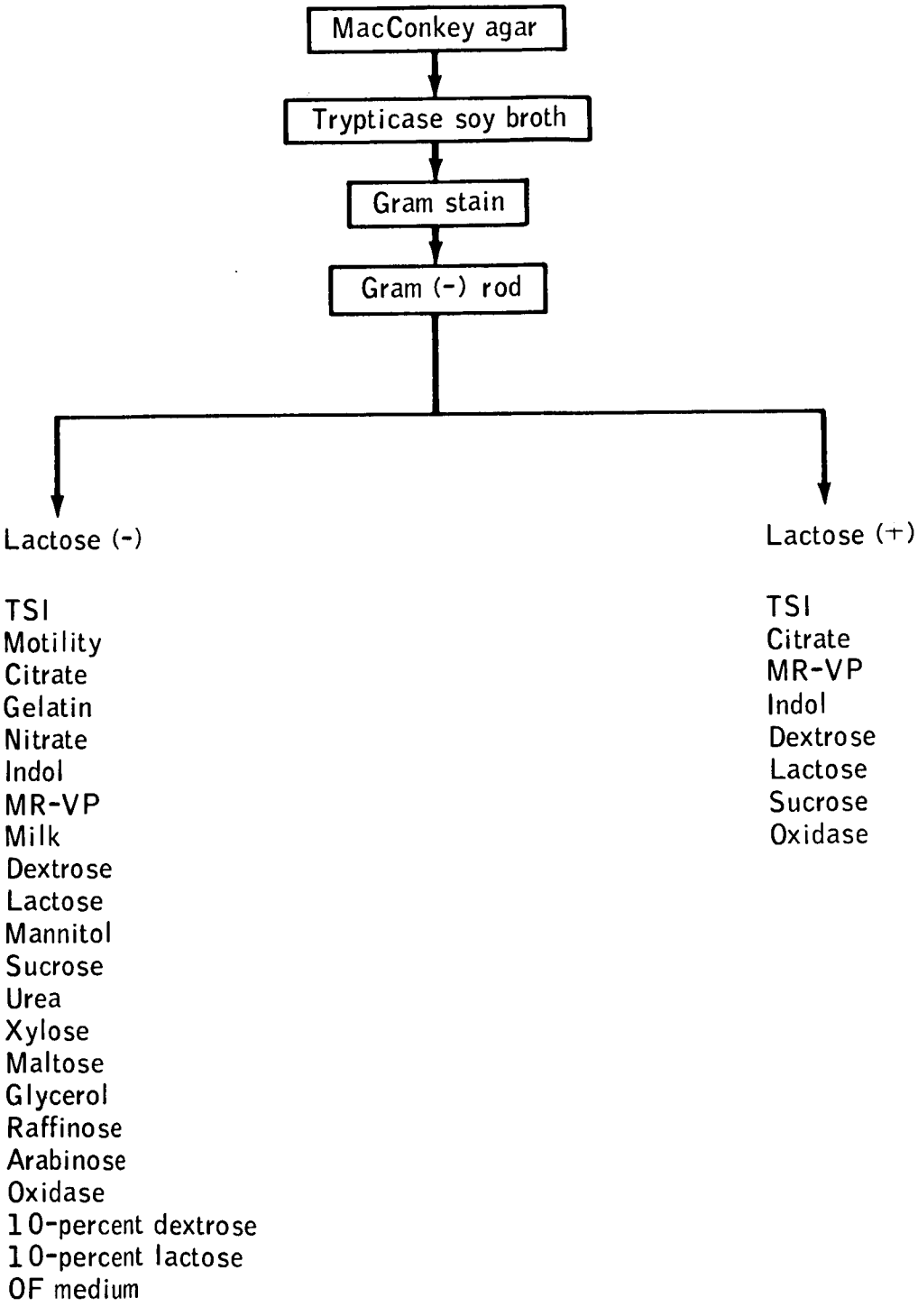


Figure 3.2.1-8.- MacConkey agar scheme.

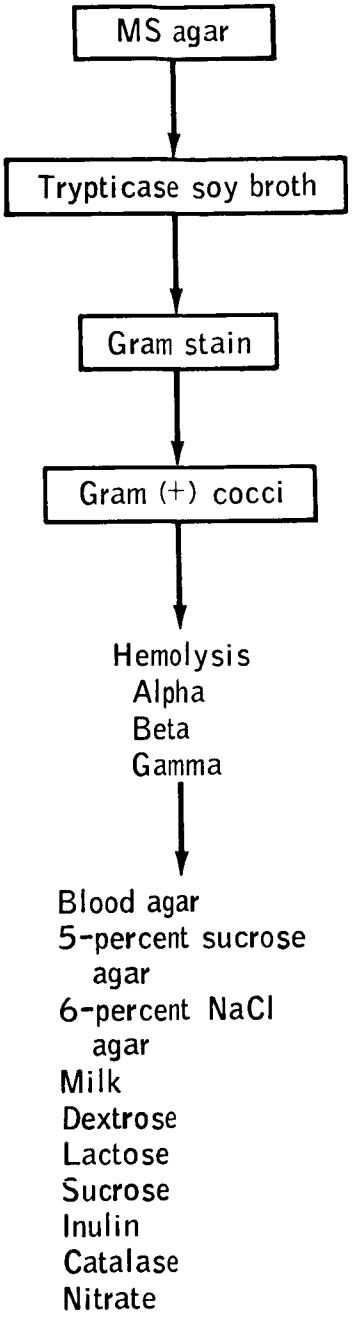


Figure 3.2.1-9.- Mitis Salivarius agar scheme.

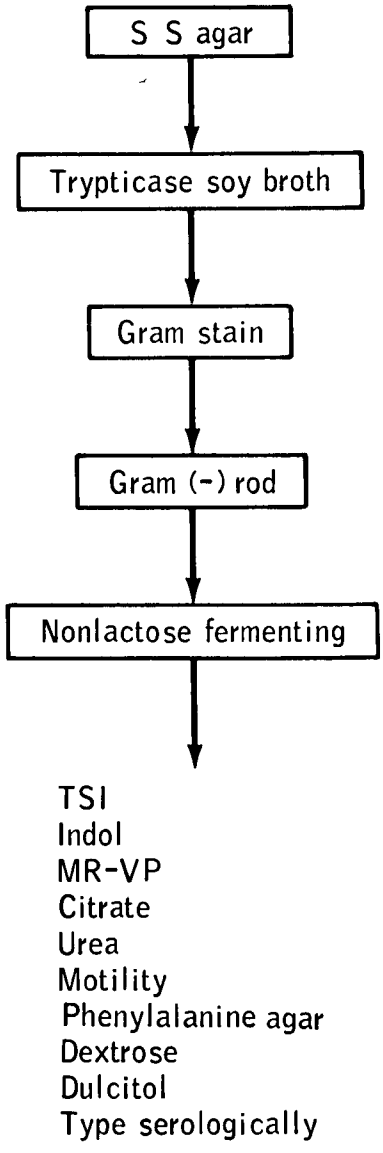


Figure 3.2.1-10.- Salmonella-Shigella agar scheme.

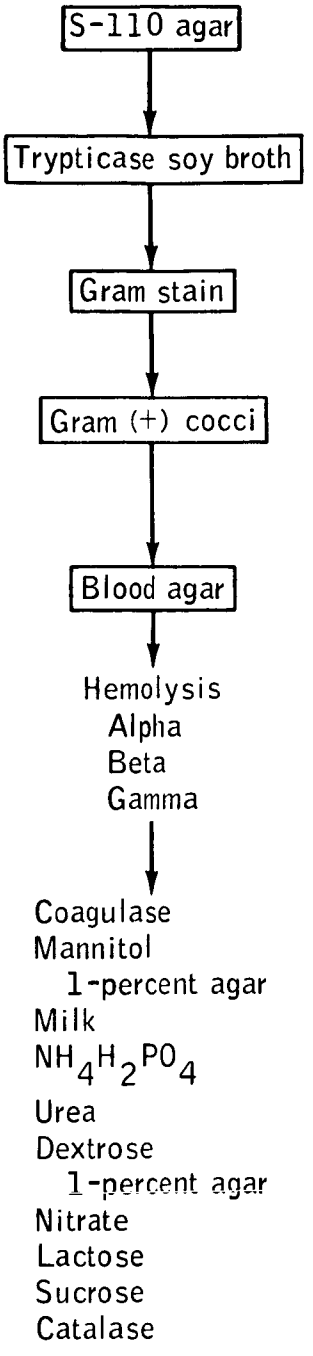


Figure 3.2.1-11.- Staphylococcus-110 agar scheme.

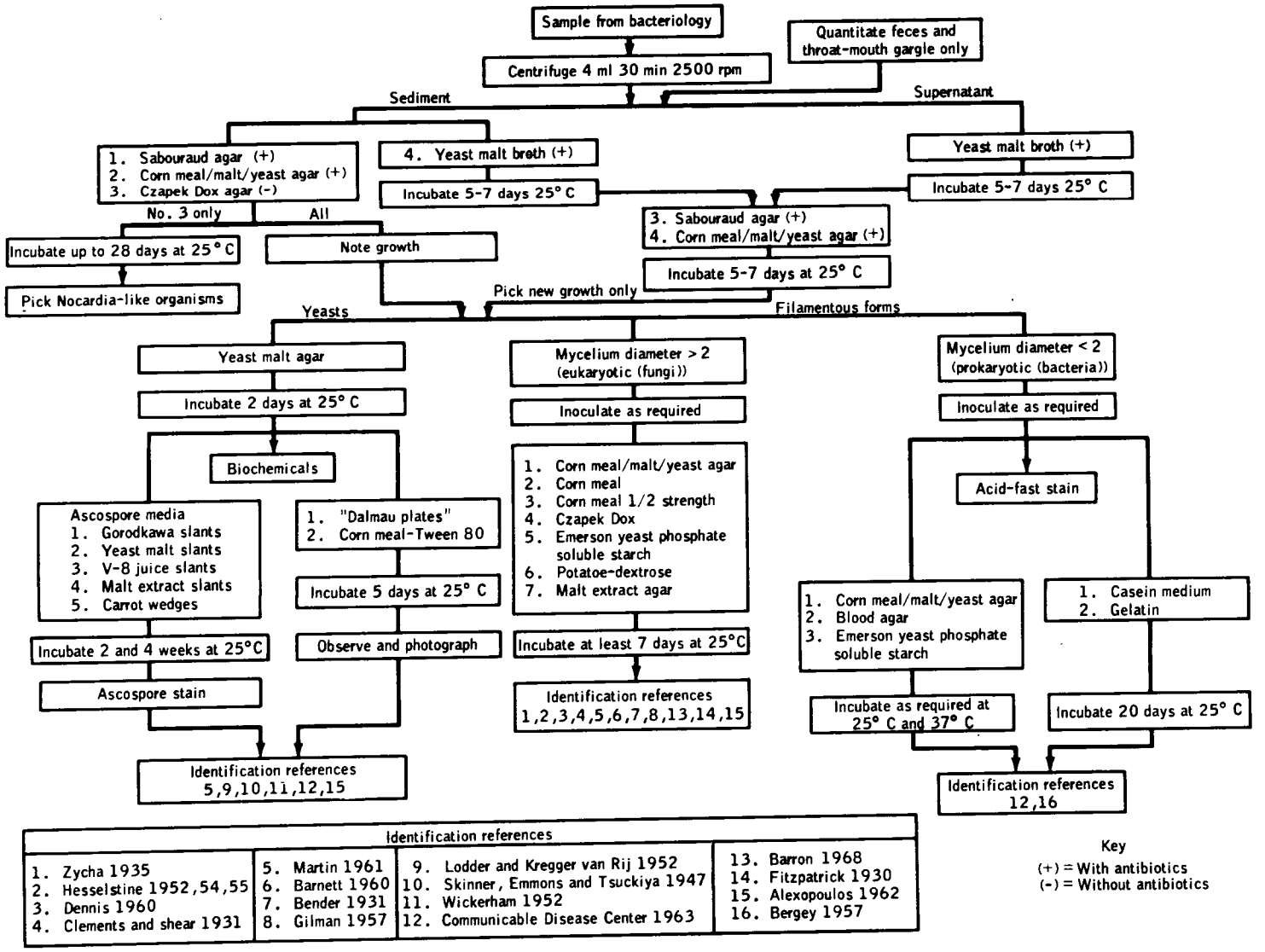


Figure 3.2.1-12.- Mycology protocol.

e. The swab will be used to streak each of the following isolation media.

(1) CMMY (containing antibiotics)

(2) SDA (containing antibiotics)

(3) CD

f. After the plates just mentioned are streaked, the swab is broken off into a second YM broth tube containing antibiotics.

g. The streaked plates and the two YM broths will be incubated at 25° C for 120 to 144 hours, at which time all colonies will be picked from the CMMY, SDA, and CD. At the end of 120 to 144 hours incubation, all YM broths are streaked onto CMMY and on SDA with antibiotics. These plates are incubated at 25° C for 120 to 144 hours. If any colonies appear that are different from these isolated in the original plates, they are picked off and identified according to the outline following.

3.2.1.4.2 Feces.-

a. One-tenth-milliliter aliquots will be removed from the 10^1, 10^2, 10^3, and 10^4 TSB stool-dilution tubes and transferred to each of the following quantitative media.

(1) CMMY (containing antibiotics)

(2) SDA (containing antibiotics)

(3) CD

b. The plates will be spread with a glass rod and incubated at 25° C for 120 hours.

c. Four milliliters of the 10^1 TSB stool-dilution tubes will be transferred aseptically to a sterile centrifuge tube. The samples will be centrifuged at 2500 rpm for 15 minutes.

d. The supernatant will be poured into a tube of YM broth (containing antibiotics) and a swab used to sample the bottom of the centrifuge tube.

e. The swab will be used to streak each of the following isolation media.

(1) CMMY (containing antibiotics)

(2) SDA (containing antibiotics)

(3) CD

f. The swab will be broken off into a second tube of YM broth containing antibiotics.

g. The streaked plates and the two YM broths will be incubated at 25° C for 120 hours.

3.2.1.4.3 External auditory canal, hands, nasal passages, and spacecraft-clothing samples (floor, maneuvering knob, drink gun, urine collection device, gloves, and shoe soles).-

a. Four milliliters of the crew external, spacecraft hardware, and clothing, 10^1 TSB dilution tubes will each be transferred aseptically to a sterile centrifuge tube. The sample will be centrifuged at 2500 rpm for 15 minutes.

b. The supernatant will be poured into 10 ml of YM broth (containing antibiotics) and a swab used to sample the bottom of the centrifuge tube.

c. The swab will be used to streak each of the following isolation media.

(1) CMMY (containing antibiotics)

(2) SDA (containing antibiotics)

(3) CD

d. The swab will be broken off into 10 ml of YM broth containing antibiotics.

e. The streaked plates and the two YM broths will be incubated at 25° C for 120 hours.

3.2.1.4.4 Urine.-

a. Four milliliters of the undiluted urine samples will be transferred aseptically to a sterile centrifuge tube. The sample will be centrifuged at 2500 rpm for 15 minutes.

b. The supernatant will be poured into 10 ml of YM broth (containing antibiotics) and a swab will be used to sample the bottom of the centrifuge tube.

c. The swab will be used to streak each of the following isolation media.

(1) CMMY (containing antibiotics)

(2) SDA (containing antibiotics)

(3) CD

d. The swab will be broken off into 10 ml of YM broth containing antibiotics.

e. The streaked plates and the two YM broths will be incubated at 25° C for 120 hours.

3.2.1.4.5 Identification.- Mycological species isolated from the throat-mouth gargle and the feces will be quantitated when feasible. All mycological species isolated will be identified according to the outline presented in figure 3.2.1-12.

3.2.1.5 Bacteriology Equipment

Room 1-125 (West)

1. Incubators, 4
2. Lamp
3. Anaerobic bags
4. TT racks
5. Pasteur pipettes

6. Pipette, 10 ml
7. Propipette
8. Pipette bulb
9. Diamond-point pencil
10. Wax pencil
11. Autoclave tape
12. Masking tape
13. Microscope
14. Microscope slides
15. Discard pan
16. Discard can
17. Trash box
18. Anaerobic indicator
19. Gram-stain material
20. Biochemical reagents
21. Paper towels
22. Sponge
23. Cloth towels
24. Scissors
25. Gas Paks
26. Felt pencil
27. Hypochlorite bottle
28. Water bottle
29. Vortex mixer
30. Hotplate

31. Cel-fibers
32. Vaspar
33. Immersion oil
34. Micro-incinerator
35. Loops
36. Filter paper
37. Animal cages
38. Stack-a-shelves
39. Forceps
40. Syringe
41. Labels

Room 1-109 (West)

1. Test tube racks
2. Spreaders (glass)
3. Turntables
4. Pipettes, 2 ml
5. Propipettes
6. Discard pans
7. Gas packs
8. Scissors
9. Forceps
10. Water bottle
11. Syringe
12. Anaerobic bags

13. Trays
14. Slides and slide holders
15. Gram stains
16. Gram-stain rack
17. Diamond-point pencils
18. Pens, pencils, wax marking pencils
19. Pasteur pipettes, short and long
20. Balance
21. Water bath, thermometer, and water
22. Hotplate
23. Weighing paper
24. Filter paper
25. Vortex mixer
26. Grippers
27. Kimwipes
28. Paper towels
29. Immersion oil
30. Hypochlorite
31. Loops
32. Micro-incinerator
33. Bulbs
34. Reagents
35. Vaspar
36. Labels

37. Millipore filter and media
38. Millipore syringe
39. Capillary tubes
40. Graduated beaker
41. Sterile bottles
42. Steroscope and light supply
43. Microscope
44. Autoclave tape
45. Tin cans
46. Shelves
47. Lacy trains
48. Spatulas
49. Sponges
50. Sterile caps
51. Sterile Petri plates
52. Lamps

3.2.1.6 Mycology Equipment

Room 1-122 (West)

1. Centrifuge, 1
2. Dissecting scope and light source, 1
3. Compound scope and camera, 1
4. Incinerator burner, 3
5. Lamp

6. Jack for dissecting scope
7. Discard pans, 4
8. Tongs
9. Variety of loops, needles, and so forth
10. Slides
11. Cover slips
12. Lactopheonol cotton-blue
13. Racks, 48 hole
14. Pipettes, 1 ml, 1 can
15. Propipette, 2
16. Pipettes, Pasteur, approximately 75
17. Immersion oil
18. Markers
19. Labels
20. Swabs
21. Spreaders, 30
22. Sterile cover slips, 1 Petri dish
23. Forceps, large and small
24. Towels, paper
25. Ascospores stains
26. Media

3.2.2 Bacteriology and Mycology Protocol for Prime and Pooled Lunar Samples

3.2.2.1 Objectives

a. To isolate and identify microorganisms in lunar samples by growth on culture media

b. To determine if the isolates are contaminants from terrestrial sources including crewmembers, spacecraft, or hardware

3.2.2.2 Protocols (Figs. 3.2.2-1 and 3.2.2-2)

3.2.2.2.1 Test design.-

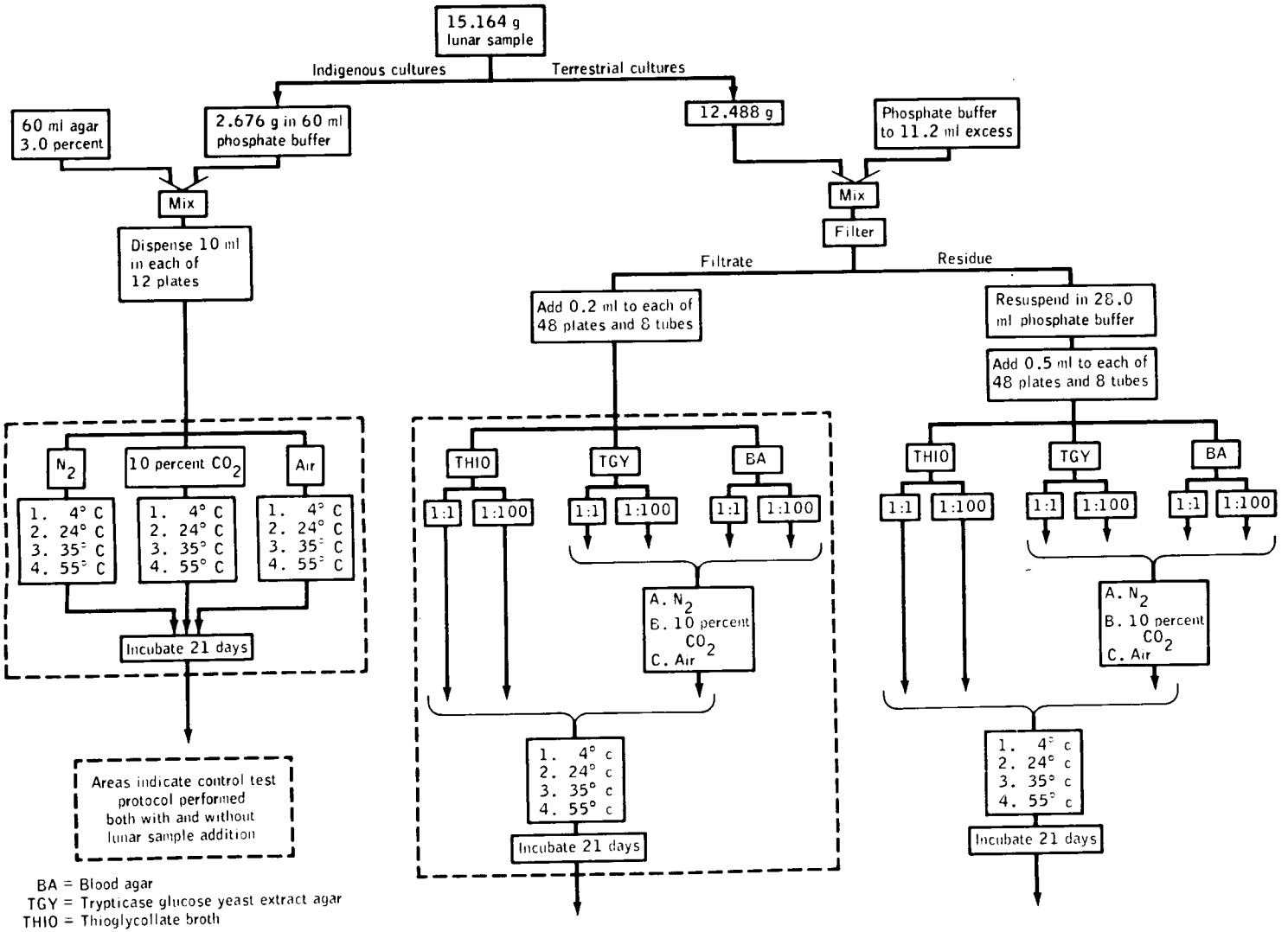
a. Four different media (indigenous agar, blood agar, trypticase glucose yeast extract agar, and thioglycollate broth) will be inoculated with prepared prime lunar sample.

b. Seventeen different media (indigenous agar, blood agar, trypticase glucose yeast extract agar, thioglycollate broth, Czapek Dox agar (CD), Sabouraud dextrose agar (SDA), pooled terrestrial-soil-extract agar, and six aquatic media) will be inoculated with prepared pooled lunar samples.

c. The media will be prepared in two different dilutions, and will be incubated at four temperatures and under three different atmospheric conditions. During incubation, the media will be observed for signs of growth. All isolates will be identified and compared to crew and hardware flora. All operations will be performed inside Class III Cabinetry in the Sample Laboratory, room 1-123.

3.2.2.2.2 Sample requirements.-

a. Approximately 0.223 g of lunar sample, sample extract, or sample residue will be used to inoculate each culture plate and culture tube.



3.2.2-1.- Microbiology protocol for prime lunar sample.

Key:

Dilutions - undiluted, 1/100

BA - Blood agar
TGY - Trypticase glucose
yeast extract agar
THIO - Thioglycollate broth
CD - Czapek Dox agar
SDA - Sabouraud dextrose agar

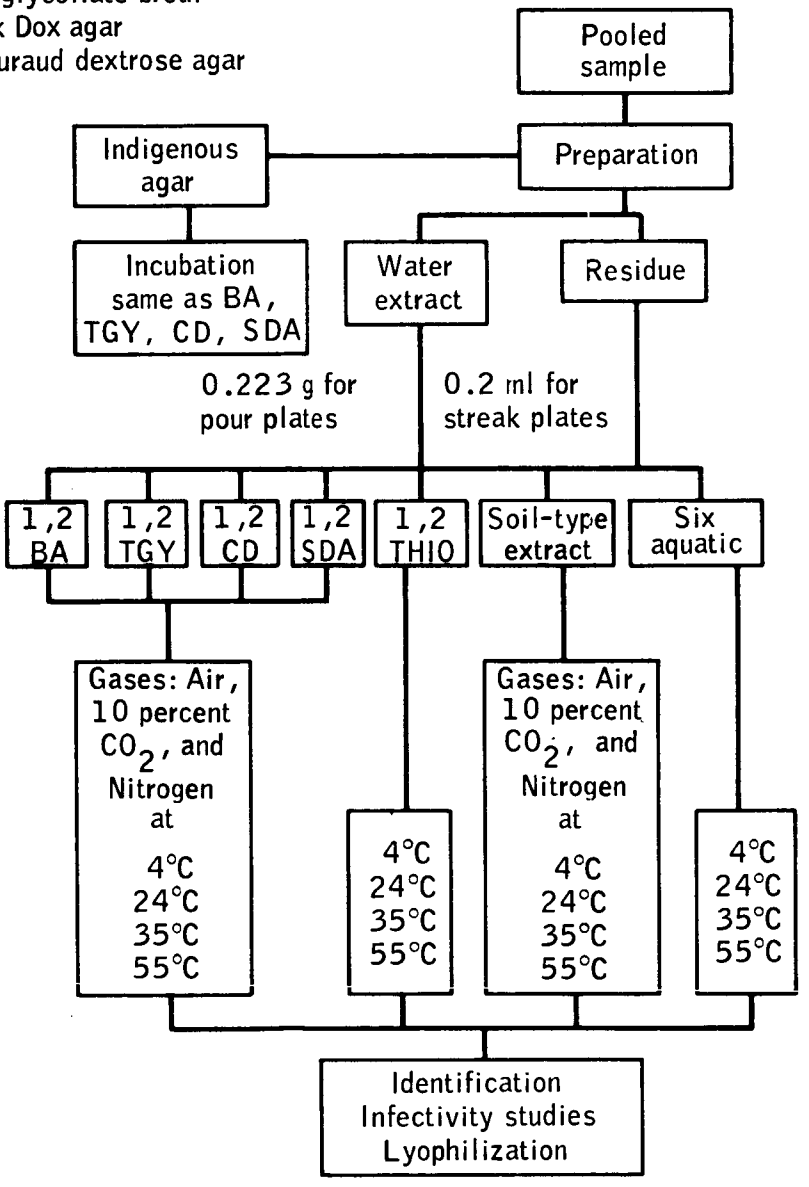


Figure 3.2.2-2.- Microbiology protocol for pooled lunar sample.

b. Prime sample: One hundred twenty-four culture plates and culture tubes will require 15.164 g of prime lunar material.

c. Pooled sample: Two hundred fifty-six culture plates and tubes will require 29.882 g of pooled lunar material.

3.2.2.2.3 Media preparation.- Media ingredients will be prepared in the support area (room 144) and autoclaved into Class III Cabinetry (room 1-123). Heat labile and sterile materials, contained in airtight bottles, will be passed into the Class III Cabinets through the autoclaves with ethylene oxide sterilization during the passage. Alternatively, sterile materials in sealed containers may be passed in through dunk tanks. The quantities described below are those required for a prime or a pooled sample.

a. Indigenous agar (prime or pooled)

(1) A total of 125 ml of double-strength purified agar (3.0 percent) will be prepared in deionized water.

(2) Five milliliters of 3.0 percent agar will be dispensed into each of 24 150- by 20-mm screwcap tubes and autoclaved for 20 minutes at 15 pounds autoclave pressure in the Class III Cabinetry.

(3) The agar preparations will be passed into the Class III Cabinetry and cooled to 50° C in a water bath.

(4) Five-milliliter aliquots of sterile phosphate buffer containing 0.223 g of prime or pooled lunar material will be added to and mixed with each of 12 tubes of the agar preparation.

(5) Sixty milliliters of phosphate buffer contained in an Erlenmeyer flask will be autoclaved for 30 minutes at 15 pounds pressure in an autoclave of the Class III Cabinetry, then cooled to room temperature within the cabinet.

(6) Five-milliliter aliquots of the sterile phosphate buffer will be added to and mixed with each of the remaining 12 tubes of the agar preparation. These 12 tubes will serve as controls.

(7) Each of the 10.0-ml mixtures (12 sample and 12 control) will be poured into a separate 60- by 15-mm sterile glass Petri plate and allowed to harden.

b. Blood agar (prime or pooled): Blood base (BioQuest) containing 1.5 percent trypticase, 0.5 percent phytone, and 0.5 percent sodium chloride will be prepared and diluted; 1.5 percent agar will be added to each aliquot of this mixture and it will be autoclaved. After cooling, blood will be added. Approximately 45 plates of each dilution (undiluted and 1/100) will be prepared.

(1) Blood base (approximately 505 ml total) will be prepared in an Erlenmeyer flask with deionized water.

(2) Five hundred milliliters of the mixture will be maintained as the undiluted medium.

(3) Four hundred and ninety-five milliliters of deionized water will be used to dilute 5.0 ml of blood base for the 1/100 dilution.

(4) Seven and one-half grams of purified agar will be added to each of the 500-ml aliquots. The flasks will be plugged with gauze and cotton.

(5) The aliquots will be transferred to the Class III Cabinet autoclave and sterilized for 15 minutes at 15 pounds pressure and at 121° C.

(6) The blood agar base aliquots will be passed into the Class III Cabinetry and cooled to 50° C in a water bath.

(7) Sixty milliliters of sterile sheep blood, contained in an airtight bottle, will be passed into the cabinet through the dunk tank containing a solution of sodium hypochlorite with 5000 ppm available Cl^-. A contact time of 30 minutes will be employed.

(8) The undiluted BA media will be prepared by adding 25 ml of blood to 450 ml of base. The 1/100 dilution will be prepared by adding 0.25 ml of blood to 499.5 ml of base. Each dilution will be swirled.

(9) Approximately two-thirds of each blood-agar dilution will be dispensed in 10.0-ml aliquots into separate 60- by 15-mm sterile glass Petri plates and allowed to harden for subsequent surface inoculation and controls. The remaining third of each dilution will be maintained at 50° C until 10.0-ml aliquots of each can be dispensed into sterile glass Petri plates containing lunar residue.

c. Trypticase glucose yeast-extract agar (TGY, BioQuest): (prime or pooled) trypticase, 0.5 percent; glucose, 0.1 percent; beef extract, 0.3 percent; and yeast extract, 0.5 percent will be prepared and diluted. One and one-half percent agar will be added to each aliquot. Approximately 45 plates of each dilution (undiluted and 1/100) will be prepared.

(1) TGY agar base (approximately 505 ml) will be prepared in an Erlenmeyer flask with deionized water.

(2) Five hundred milliliters of the mixture will be maintained as the undiluted medium.

(3) Four hundred ninety-five milliliters of deionized water will be used to dilute 5.0 ml of TGY agar base for the 1/100 dilution.

(4) Seven and one-half grams of purified agar will be added to each of the 1.0-liter aliquots. The flask will be plugged with gauze and cotton.

(5) The aliquots will be transferred to the Class III Cabinet autoclave and sterilized for 20 minutes at 15 pounds pressure and at 121° C.

(6) The TGY aliquots will be passed into the Class III Cabinetry and cooled to 50° C in a water bath.

(7) Approximately two-thirds of each TGY agar dilution will be dispensed in 10.0-ml aliquots into separate 60- by 15-mm sterile glass Petri plates and allowed to harden for subsequent surface inoculation and/or controls.

(8) The remaining third of each dilution will be maintained at 50° C until 10.0-ml aliquots of each can be dispensed into sterile glass Petri plates containing lunar residue.

(9) A total of 90 TGY agar plates will be prepared.

d. Thioglycollate broth: (prime or pooled) Thioglycollate broth (BioQuest) containing yeast extract, 0.5 percent; casitone, 1.5 percent; dextrose, 0.5 percent; sodium chloride, 0.25 percent; L-cystine, 0.025 percent; thioglycollic acid, 0.03 percent; and resazurin, 0.0001 percent will be prepared and diluted. Seventy-five thousandth percent of purified agar will be added to each aliquot. Approximately 28 tubes of each dilution (undiluted and 1/100) will be prepared.

(1) Approximately 505 ml of thioglycollate broth will be prepared in an Erlenmeyer flask with deionized water.

(2) Five hundred milliliters of the broth will be maintained as the undiluted medium.

(3) Four hundred ninety-five milliliters of deionized water will be used to dilute 5.0 ml of thioglycollate broth for the 1/100 dilution.

(4) Three hundred seventy-five milligrams of purified agar will be added to each 500.0-ml aliquot.

(5) Twenty-milliliter aliquots of each broth dilution will be dispensed into each 150- by 20-mm screwcap test tube, making a total of 50 screwcap test tubes.

(6) The tubes will be transferred to the Class III Cabinet autoclave and sterilized for 20 minutes at 15 pounds pressure and at 121° C.

(7) The tubes will be passed into the Class III Cabinetry and allowed to cool.

e. Czapek Dox agar: (pooled only) Czapek Dox (Difco) medium containing saccharose, 3.0 percent; sodium nitrate, 0.2 percent; dipotassium phosphate, 0.1 percent; magnesium sulfate, 0.05 percent; potassium chloride, 0.05 percent; and ferrous sulfate, 0.001 percent will be prepared and diluted. One and one-half percent agar will be added to each aliquot. Approximately 36 plates of each dilution (undiluted and 1/100) will be prepared.

(1) Approximately 500.0 ml of CD will be prepared in Erlenmeyer flasks with deionized water.

(2) Four hundred fifty milliliters of the mixture will be maintained as the undiluted medium.

(3) Four hundred and forty-five milliliters of deionized water will be used to dilute 4.5 ml of CD medium for the 1/100 dilution.

(4) Six and seventy-five hundredths grams of purified agar will be added to each 450.0-ml aliquot. The flasks will be plugged with gauze and cotton.

(5) The media will be transferred to the Class III Cabinet autoclave and sterilized for 20 minutes at 15 pounds pressure and at 121° C.

(6) The media will be passed into the Class III Cabinetry and cooled to 50° C in a water bath.

(7) Approximately two-thirds of each CD agar dilution will be dispensed in 10.0-ml aliquots into separate 60- by 15-mm sterile glass Petri plates and

allowed to harden for subsequent surface inoculation and/or controls.

(8) The remaining third of each dilution will be maintained at 500° C until 10.0-ml aliquots of each dilution can be dispensed in sterile glass Petri plates containing lunar residue.

(9) A total of 72 CD agar plates will be prepared.

f. Sabouraud dextrose agar: (pooled only) Sabouraud dextrose medium (Difco) containing neopeptone, 1.0 percent; and dextrose, 4.0 percent will be prepared and diluted. One and one-half percent agar will be added to each aliquot. Approximately 36 plates of each dilution (undiluted and 1/100) will be prepared.

(1) Approximately 500.0 ml of Sabouraud dextrose medium will be prepared in Erlenmeyer flasks with deionized water.

(2) Four hundred fifty milliliters of the mixture will be maintained as the undiluted medium.

(3) Four hundred forty-five milliliters of deionized water will be used to dilute 4.5 ml of SDA medium for the 1/100 dilution.

(4) Six and seventy-five hundredths grams of purified agar will be added to each 450.0-ml aliquot. The flasks will be plugged with gauze and cotton.

(5) The media will be transferred to the Class III Cabinet autoclave and sterilized for 20 minutes at 15 pounds pressure and at 121° C.

(6) The media will be passed into the Class III Cabinetry and cooled to 50° C in a water bath.

(7) Approximately two-thirds of each SDA agar dilution will be dispensed in 10.0-ml aliquots into separate 60- by 15-mm sterile glass Petri plates and allowed to harden for subsequent surface inoculation and/or controls.

(8) The remaining third of each dilution will be maintained at 50° C until 10.0-ml aliquots of each dilution can be dispensed into sterile glass Petri plates containing lunar residue.

(9) A total of 72 SDA agar plates will be prepared.

g. Terrestrial-soil extract: (pooled only) Five different soil samples will be pooled, extracted (Cameron, Space Programs Summary No. 37-38, Vol. IV, 1966), mixed with agar, sterilized, and incorporated into Petri plates (table 3.2.2-I).

(1) Twenty-five grams of soil sample will be added to 500.0 ml of sterile deionized water at least 2 days before needed.

(2) The material will be homogenized at low speed in a Waring blender for 5 minutes.

(3) The soil sample will be extracted further by standing for 18 hours in the same solution at a temperature indigenous to the Class III Cabinetry.

(4) The extract will be filtered through Whatman No. 3 filter paper and collected in a 500.0-ml volumetric flask.

(5) The residue contained in the filter will be washed with sterile deionized water until the total supernate volume is 500.0 ml.

(6) Seven and five-tenths grams of purified agar will be added to 500.0 ml of each soil extract in an Erlenmeyer flask. The flasks will be plugged with gauze and cotton.

(7) The media will be transferred to the Class III Cabinet autoclave and sterilized for 30 minutes at 15 pounds pressure and at 121° C.

(8) The media will be passed into the Class III Cabinetry and cooled to 50° C in a water bath.

TABLE 3.2.2-I.- ORIGIN OF SOILS USED IN TERRESTRIAL SOIL EXTRACT EMPLOYED IN POOLED LUNAR SOIL INOCULATION

Soil type	Sample area	Date
Tundra	Cape Royds, Alaska	1/15/68
Desert	Tucson, Arizona	10/26/67
Coniferous forest	Bastrop, Texas	1/18/69
Tropical rain forest	Republic of Panama	2/68
Prairie/grassland	Sweetwater, Oklahoma	10/7/68

(9) Approximately two-thirds of each soil-extract preparation will be dispensed in 10.0-ml aliquots into a separate 60- by 15-mm sterile glass Petri plate and allowed to harden for subsequent surface inoculation and/or controls.

(10) The remaining third of each soil preparation will be maintained at 50° C until 10.0-ml aliquots of each preparation can be dispensed into sterile glass Petri plates containing lunar residue.

(11) A total of 36 soil-extract agar plates will be prepared.

h. Aquatic media: Six aquatic and mud samples will be sterilized and dispensed in capped test tubes (table 3.2.2-II).

(1) Aquatic samples will be obtained aseptically from the designated areas.

(2) One-hundred-milliliter aliquots of each aquatic sample will be dispensed into eighteen 200- by 38-mm capped test tubes in such a manner that a layer of bottom mud is overlaid with indigenous water.

(3) The aquatic media will be transferred to the Class III Cabinet autoclave and sterilized for 30 minutes at 15 pounds pressure and at 121° C.

(4) Tubes will be passed into the Class III Cabinetry and allowed to cool.

i. Media for backup studies: The media for stock cultures and for biochemical characterization will be prepared in the media room (room 144). The media will be autoclaved into the Class III Cabinetry of room 1-123 as has been described.

3.2.2.2.4 Sterility testing of media.-

a. Incubation

(1) All media preparations will be transferred to 35° C incubators located in room 1-123 Class III Cabinet.

TABLE 3.2.2-II.- WATER AND MUD SAMPLES USED FOR POOLED LUNAR-SOIL INOCULATIONS

Date	General location	Specific location
3/5/68	Gulf of Mexico	100 miles off Galveston, Texas (720 meters)
5/31/69	Recovery zone	Off Molokai Island, Hawaii (1.5 meters)
1/18/69	Cold-water lake	Barton Springs, Austin, Texas
1/18/69	Warm-water lake	Town Lake, Austin, Texas
Open	Hot Springs	Open
Open	Arctic Ocean	Open

(2) The media will be incubated for 48 hours.

b. Observations

(1) The media preparations will be observed at 24 and 43 hours for signs of contamination.

(2) All contaminated plates will be discarded by autoclaving out of the Class III Cabinetry.

3.2.2.2.5 Preparation of lunar material.-

a. Indigenous cultures (Fig. 3.2.2-3)

(1) The lunar sample (2.676 g) will be added to a sterile medicine bottle containing 60.0 ml of sterile phosphate buffer. Phosphate buffer will be made as follows.

(a) 100 ml of 0.1M KH_2PO_4

(b) 58.2 ml of 0.1M NaOH

(c) pH = 7.0

(2) The mixture will be homogenized by hand for 1 minute.

(3) Five milliliters of the mixture will contain 0.223 g of lunar material.

b. Prime sample (Fig. 3.2.2-4)

The following procedures will be employed for lunar material of fine particle size (less than 104μ).

(1) The prime sample (12.488 g) will be added to a sterile glass bottle containing 12.0 ml of sterile phosphate buffer.

(2) The contents will be mixed by hand for 1 minute.

(3) The mixture will be filtered through washed, sterilized, Whatman No. 3 filter paper resting on a Buckner funnel; 11.2 ml of extract will be collected in a sterile glass beaker. Additional aliquots of phosphate buffer will be used to

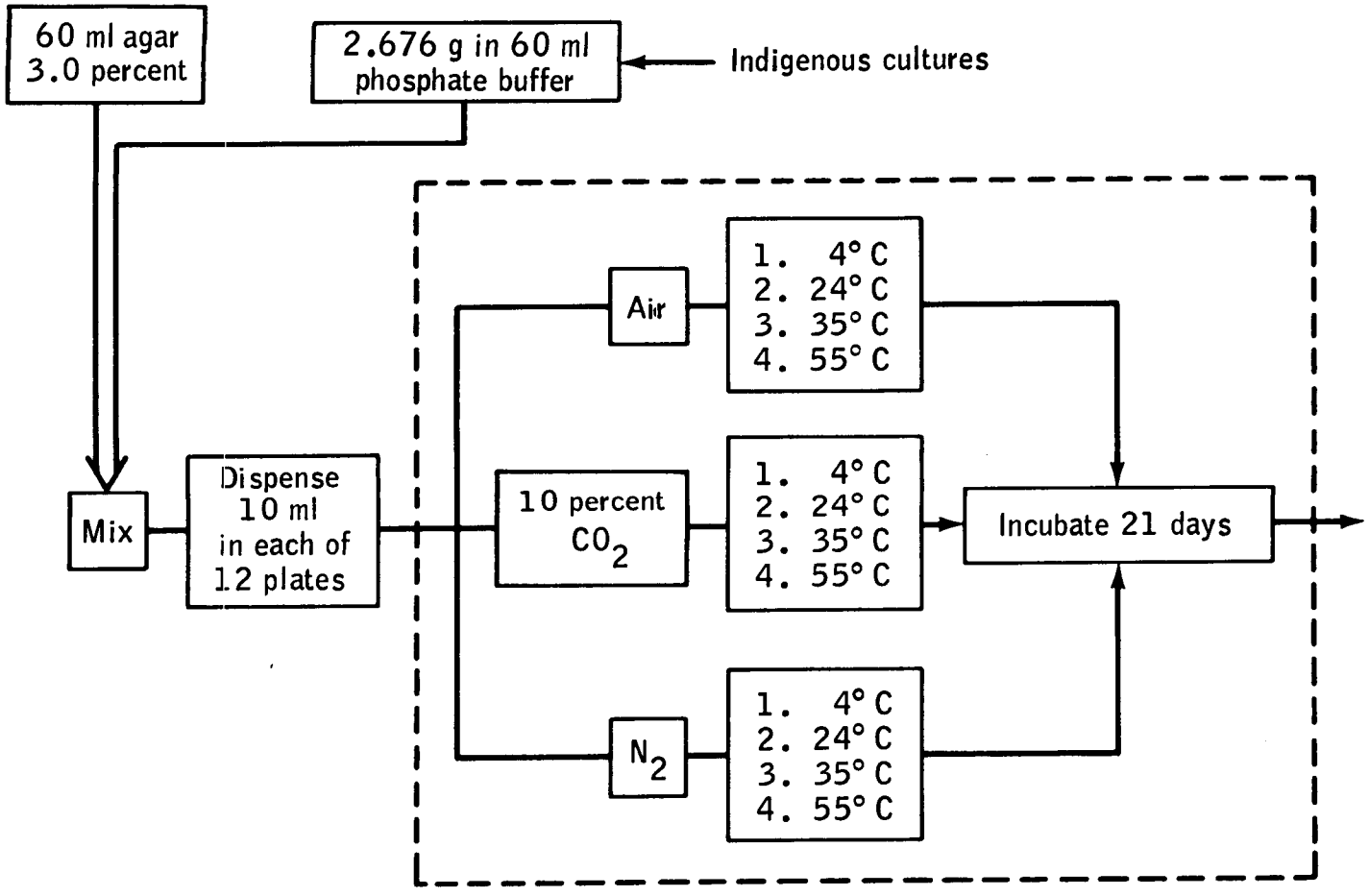


Figure 3.2.2-3.- Microbiology protocol for prime lunar sample, indigenous cultures.

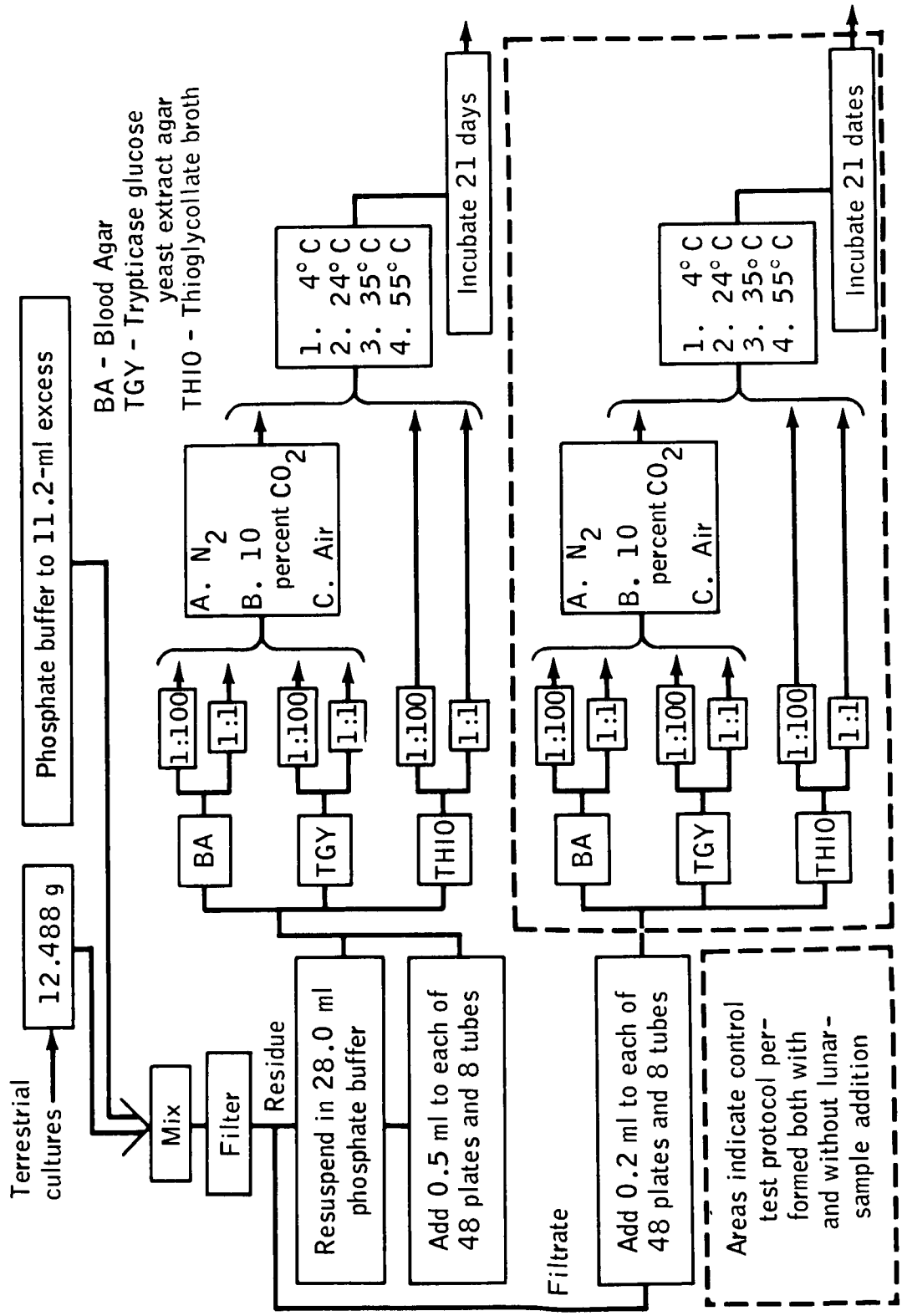


Figure 3.2.2-4.- Microbiology protocol for prime lunar sample, terrestrial cultures.

wash the residue in order to meet the 11.2-ml requirement.

(4) The supernatant of the prime-sample extract will be used to inoculate one set of prime-sample media (BA, TGY agar, and thioglycollate broth).

(5) The residue of the extracted prime sample will be incorporated into one set of prime-sample media (BA, TGY agar, and thioglycollate broth).

c. Pooled sample: The following procedures will be employed for lunar material of fine particle size (less than 104μ).

(1) The pooled sample (27.206 g) will be added to a sterile glass bottle containing 28.0 ml of sterile phosphate buffer.

(2) The contents will be mixed by hand for 1 minute.

(3) The mixture will be filtered through washed, sterilized Whatman No. 3 filter paper resting on a Buchner funnel.

(4) Twenty-four and four-tenths milliliters of extract will be collected in a sterile glass beaker.

(5) Additional aliquots of phosphate buffer will be used to wash the residue in order to meet the 24.4-ml requirement.

(6) The supernatant of the sample extract will be used to inoculate one set of media (BA, TGY agar, thioglycollate broth, CD agar, SDA agar, terrestrial-soil-extract agar, and six aquatic media).

(7) The residue of the extracted sample will be incorporated into one set of sample media (BA, TGY agar, thioglycollate broth, CD agar, SDA agar, terrestrial-soil-extract agar, and six aquatic media).

3.2.2.2.6 Inoculation of media.- The prepared lunar-sample extracts and the residues will be pipetted onto or incorporated into each designated medium.

a. Lunar extract inoculation

(1) The top from each Petri plate containing hardened media will be removed.

(a) A propipette will be used to transfer 0.2 ml of lunar extract to each medium; 0.2 ml of extract will represent the washing of 0.223 g of lunar material.

(b) The lunar extract will be spread evenly over the surface of the medium with a glass spreader.

(c) The top of each Petri plate will be replaced.

(2) The screwcap from each tube of broth media will be removed (thioglycollate broth and aquatic medium)

(a) A propipette will be used to transfer 0.2 ml of lunar extract to each fluid medium (thioglycollate broth and aquatic medium).

(b) The screwcap from each tube will be replaced.

b. Lunar residue inoculation

(1) Media will each be maintained in a water bath at 50° C.

(2) The lunar residue contained on the Whatman No. 3 filter paper will be transferred to a sterile glass beaker.

(3) Twenty-eight milliliters of sterile phosphate buffer will be used to wash the prime lunar residue from the filter paper into the glass beaker.

(4) Sixty-eight milliliters of sterile phosphate buffer will be used to wash the pooled lunar residue from the filter paper into the glass beaker.

(5) A sterile calibrated Pasteur pipette will be used to transfer 0.5 ml of lunar soil to each empty Petri plate; 0.5 ml will contain 0.223 g of lunar material.

(6) The top of each 60- by 15-mm sterile glass Petri plate will be removed and the aliquots of lunar residue material will be transferred to each plate.

(7) Ten-milliliter aliquots of the designated media will be poured into each Petri plate containing the lunar residue, and the top of each plate will be replaced.

(8) Each plate will be swirled 10 times in a rotary fashion and allowed to harden.

(9) The screwcap from each tube of broth media will be removed (thioglycollate broth and aquatic medium).

(10) The samples of lunar residue material will be transferred to each tube.

(11) The screwcap from each tube will be replaced.

c. Controls

(1) Petri plates filled with media will be incubated within controlled-environment enclosures as are uninoculated controls.

(a) One hundred and twenty plates for the pooled sample

(b) Sixty plates for the prime sample

(2) The media will be incubated under test atmospheres and at test temperatures.

(3) Screwcap tubes containing broth media will be incubated at test temperatures as uninoculated controls.

(a) 14 tubes for pooled sample

(b) 8 tubes for prime sample

(4) All control media and test media will be poured into aliquots within Class III Cabinetry.

3.2.2.2.7 Incubation.- Inoculated Petri plates will be placed in controlled-environment enclosures and the test-atmospheric requirements met. The enclosures will be passed to incubators set at test temperatures.

a. Placement into controlled-environment enclosures

(1) The back plate from each enclosure will be removed.

(2) Two or three inoculated glass Petri plates will be placed into each enclosure (tables 3.2.2-III and 3.2.2-IV).

(3) Control media will be enclosed in a similar fashion.

(4) The backplate from each enclosure will be replaced and firmly fastened.

b. Atmospheric requirements

(1) The outlet port of each enclosure will be opened.

(2) The inlet port of each enclosure will be attached to the appropriate gas port.

(3) Aerobic requirements will be met with filtered air from the cabinet lines.

(4) Microaerophilic requirements will be met with a mixture of 10.0 percent CO_2 in air.

(5) Anaerobic requirements will be met with dry, sterile, prepurified nitrogen.

TABLE 3.2.2-III.- PLACEMENT OF PETRI PLATES IN CONTROLLED-ENVIRONMENT ENCLOSURES, PRIME LUNAR SAMPLE

Code	Temp, °C	Atmosphere	Group 1	Group 2	Group 3 (a)	Group 4 (a)
A B C D	4 24 35 55	Air	BA/BA/IND 100	TGY/TGY/AG 100	BA/BA/0 100	TGY/TGY/0 100
E F G H	4 24 35 55	CO_2				
I J K L	4 24 35 55	N_2				

[a]Two series of experimental plates are used: (1) Inoculated with lunar extract and (2) Inoculated with lunar residue. A total of 72 controlled-environmental enclosures are used.

Note: BA = Blood agar

TGY = Trypticase glucose yeast extract agar

IND = Lunar soil + phosphate buffer + agar

AG = Phosphate buffer + agar (uninoculated control)

TABLE 3.2.2-IV.- PLACEMENT OF PETRI PLATES IN CONTROLLED-ENVIRONMENT ENCLOSURES, POOLED SAMPLE

Code	Temp, °C	Atmosphere	Group 1 (a)	Group 2 (a)	Group 3 (a)	Group 4 (a)
AA BB CC DD	4 24 35 55	Air	BA/BA/SAB 100	TGY/TGY/SAB 100 100	CD/CD/SE 100	IND/AG/0
EE FF GG HH	4 24 35 55	CO_2				
II JJ KK LL	4 24 35 55	N_2				

[a]Groups 1, 2, and 3 apply to three different sets of media, inoculated as indicated below.

(1) Residue of lunar soil

(2) Extract of lunar soil

(3) Uninoculated control

A total of 120 controlled-environment enclosures are used.

Note: TGY = Trypticase glucose yeast extract agar

BA = Blood agar

SE = Soil extract

IND = Lunar soil + phosphate buffer + agar

AG = Phosphate buffer + agar (uninoculated control)

(6) Each enclosure will be degassed three times and gassed each time with the appropriate atmosphere.

(7) An anaerobic indicator (methylene blue) will be included in a Durham tube in each nitrogen-gassed enclosure.

c. Temperature requirements

(1) Filled enclosures and inoculated, tubed media will be passed to appropriate incubators at the end of the cabinet line.

(2) Incubators, located within the Class III Cabinetry of room 1-123, will have the following temperatures.

(a) 4° C

(b) 24° C (indigenous temperature of Class III Cabinet, room 1-123)

(c) 35° C

(d) 55° C

(e) The combined soil-extract agar will be incubated at each of the four temperatures.

(f) Incubation temperatures for the six different aquatic media are as follows.

Arctic Ocean — 4° C
Gulf of Mexico — 24° C
Recovery zone — 24° C
Cold-water lake — 4° C
Warm-water lake — 35° C
Hot spring — 55° C

3.2.2.2.8 Observation to be made.- All observations will be made within the Class III Cabinetry of room 1-123.

a. The inoculated media will be examined every 24 hours for colony formation, turbidity, and gross visual changes occuring in the media. The media will be examined under a stereomicroscope (× 4 and × 30)

incorporated into the Class III Cabinetry. All inoculated media will be compared with control media.

b. The following characteristics will be recorded daily.

(1) Length of time for growth to occur

(2) Atmospheric condition

(3) Temperature

(4) Media supporting growth

c. Thirty-five millimeter photocolor slides will be used to record colony characteristics and changes in media characteristics.

3.2.2.2.9 Labeling.-

a. Media

(1) Each Petri plate will be labeled with a code letter and number (tables 3.2.2-III and 3.2.2-IV).

(a) The code letter will be recorded in a laboratory notebook and will correspond to the letter on the controlled environment enclosure. Letter information will reveal the following.

1. Gas(es) used

2. Temperature of incubation

(b) The number will be recorded in the laboratory notebook as a subunit of the letter designation. Number information will reveal the following.

1. Media type

2. Dilution

3. Preparation data

4. Test or control

5. Prime or pooled sample number

6. Extract or residue

7. Date of inoculation

(2) Each screwcap test tube will be labeled with a number only. The number will be recorded in the laboratory notebook.

Number information will reveal the following.

(a) Media type

(b) Dilution

(c) Preparation date

(d) Test or control

(e) Prime or pooled sample number

(f) Extract or residue

(g) Date of inoculation

(h) Temperature

b. Controlled environment enclosures

The code letter will be recorded in a laboratory notebook and will correspond to the letter on the contained Petri plates.

c. Recording data

(1) A logbook will be used to record all operations.

(2) One technician will record all operations performed by each technician.

(3) A 35-mm color slide will be taken of appropriate enclosures and test tube racks. All pertinent operations will be recorded on color film. Notes will be made to indicate colonies picked for further study.

3.2.2.2.10 Backup studies.-

a. Identification of isolates: All isolates will be characterized and identified by morphological, biochemical, and serological techniques.

(1) Colony characteristics will be recorded.

(2) The media supporting the organisms grown will be recorded.

(3) Hemolytic activity will be recorded.

(4) A smear from a colony will be Gram stained according to the following scheme. The stained cells will be observed at × 1000 magnification with a light microscope located in room 1-123.

(a) A smear of the material to be stained will be prepared by transferring the material to a drop of deionized water on a clean glass slide.

(b) The material will be allowed to air-dry and then the slide will be heat-fixed on a hotplate.

(c) The slide will be flooded with crystal violet for 1 minute. The crystal violet will be washed away with tap water.

(d) The slide will be flooded with iodine solution for 1 minute. The iodine will be washed away with tap water.

(e) The slide will be flooded with 95 percent ethanol for 15 seconds. The ethanol will be washed away with tap water.

(f) The slide will be flooded with Safranin for 1 minute. The Safranin will be washed away with tap water.

(g) The slide will be blotted and observed under the oil immersion objective of a microscope.

(5) A portion of each colony will be transferred to 10.0 ml of sterile trypticase soy broth.

(6) A portion of each colony will be streaked onto the same medium from which it was isolated.

(7) The trypticase soy broth and the isolation medium will be incubated at the isolation temperature and atmosphere.

(8) Gram-positive (+) rod-shaped organisms will be inoculated into TGY. Normal Gram-stain preparations will be observed for presence of spores.

(9) Gram-positive rod-shaped organisms will be acid-fast stained according to the following scheme.

(a) A smear of the material to be stained will be prepared by transferring the material to a drop of deionized water on a clean glass slide.

(b) The material will be allowed to air-dry and then the slide will be heat-fixed on a hotplate.

(c) The slide will be flooded with carbofuchsin for 4 minutes. The slide will be warmed on a hotplate during this time. The carbofuchsin will be washed away with tap water.

(d) The slide will be washed with 3 percent acid alcohol followed by a wash with water.

(e) The slide will be flooded with brilliant green for 2 minutes. The brilliant green will be washed away with tap water.

(f) The slide will be blotted dry and observed under the oil immersion objective of a microscope.

(10) The trypticase soy broth culture will be used to inoculate biochemical media required for the identification of each isolate.

b. Comparison of isolates with crewmember and spacecraft flora

(1) Each isolate will be compared with microbiological profiles of the Apollo crewmembers and clothing.

(2) Each isolate will be compared with microbiological profiles of the Apollo spacecraft.

c. Infectivity studies:

In the event that it is required, isolates will be subjected to infectivity studies in normal mice.

(1) Each isolate will be inoculated onto three slants of each media from which the organisms were isolated.

(2) The slants will be incubated for 16 hours at the temperature indigenous to the original isolation.

(3) The cells from each slant will be harvested by washing three times with sterile phosphate buffer.

(4) The harvests from each slant will be pooled and centrifuged for 10 minutes at 5000 rpm (4500 × g).

(5) The supernatant will be discarded. The sediment will be resuspended in 10.0 ml of sterile phosphate buffer diluent.

(6) The cell suspension will be diluted from 1×10^{-1} through 1×10^{-8} with sterile phosphate buffer. Pour-plate counts will be performed with isolation media on each dilution.

(7) Five normal white mice (strain C57-BL/6, 4 to 6 weeks old) will be injected intraperitoncally (IP) with 1.0 ml or the undiluted, 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, and 1×10^{-4} dilutions.

(8) One milliliter of sterile phosphate buffer will be injected into each of five control mice. In addition, five control uninjected mice will be isolated in a separate cage for each isolate tested.

3.2.2.2.11 Exit of materials from cabinetry.-

a. Autoclavable material

(1) Materials to be sterilized will be placed in the cabinet autoclave.

(2) The material will be autoclaved at 120° C and 15 pounds pressure for 30 minutes using operations procedures described in the OMV4S9 for autoclaves in the respective rooms.

(3) The material will be removed through the outside door.

b. Nonautoclavable material

(1) Materials to be sterilized will be placed in the cabinet autoclave.

(2) The material to be sterilized will be exposed to ethylene oxide gas for a period of 16 hours.

(3) The material will be removed through the outside door.

c. Dunk tank

(1) Materials will be placed in containers employing procedures described in OPV7S2.

(2) The containers will be placed in the sodium hypochlorite (5000 ppm available Cl^-) dunk tank for 15 minutes.

3.2.2.2.12 Emergency plans.-

a. Media failure

(1) A 10-percent overage of each type of media will be prepared and will be available within the Class III Cabinetry.

(2) A 100-percent overage of each type of media will be prepared in Erlenmeyer flasks and will be stored in the Sample Area.

b. Equipment failure

(1) A list of surplus minor equipment that will be available within Class III Cabinetry follows.

(a) Pipettes

(b) Inoculating tools

(c) Glass Petri plates

(d) Test-tube racks

(e) Controlled-environment enclosures

(f) Propipette bulbs

(g) Marking pencils

(h) Labels

(i) Micro-incinerators

(j) Light bulbs for microscopes

(k) Slides

(l) Staining material

(m) Tool kit

(2) A list of surplus major equipment that will be available in the Sample Laboratory follows.

(a) Water baths

(b) Microscopes

(c) Hotplate

(3) Surplus major and minor equipment will be available in the Support Laboratory.

c. Cabinet failure

(1) Alternate Class III Cabinetry in room 1-125 will be available in case of primary cabinet failure.

(2) If cabinet failure is not extensive, the cabinetry will be repaired and resterilized.

d. Technician failure

Available microbiology personnel may be employed on an overtime basis in case of a general manpower shortage.

3.2.2.3 Support Effort

a. Materials required and surplus media

(1) A supply of each type of media will be prepared in 2-liter Erlenmeyer flasks and stored at 4° C.

(2) All equipment employed in the Class III Cabinetry and in the Support Laboratory will be duplicated in the Lunar Receiving Laboratory storeroom.

b. Quality control

(1) Petri plates, pipettes, and glassware

(a) Glass Petri plates will be washed with Alconox, rinsed three times with deionized water, and dried in an oven.

(b) Pipettes and glassware will be washed with Alconox and cleaned with sulfuric acid/potassium dichromate cleaning solution. The glassware will be rinsed three times with deionized water and dried in an oven.

(2) Deionized water

(a) All media and reagents will be prepared with certified deionized water.

(b) The deionized water will be certified to contain less than 1.0 ppm of extraneous ions.

(3) Media

(a) All prepared media will be tested for sterility by incubation at 35° C for 48 hours in incubators within the Class III Cabinetry.

(b) All commercial media will be purchased in batch lots and the lot number will be checked on each new container.

(c) All prepared media will be tested for ability to support growth, and to determine the quality of colony formation of each of the following test organisms.

1. Staphylococcus aureus
2. Neisseria gonorrhea
3. Streptococcus species (β-hemolytic)
4. Aspergillus niger
5. Hemophilus influenzae
6. Escherichia coli
7. Clostridium perfringens

c. Time schedules

The applicable time schedules are presented in table 3.2.2-V.

d. Logistics

The logistics, based on one prime and one pooled sample are presented in tables 3.2.2-VI and 3.2.2-VII.

e. Pretest preparation of test area

(1) Class III Cabinetry

(a) All Class III Cabinetry will be certified for 100-percent isolation using Freon leak-detection apparatus. The methods used for certification will be described in appendix A, OPV7S2.8.

(b) All Class III Cabinetry will be sterilized using paraformaldehyde. The methods used for sterilization will be described in OPV7S2.

TABLE 3.2.2-V.- TIME SCHEDULES

Activity	Time in reference to receipt of sample in Bioprep Cabinetry, days
Class III Cabinet preparation	
1. Sterilization	-10
2. Sterility testing	- 8
Media preparation	
1. Media preparation	- 3
2. Sterilization and dispensing	- 2
3. Sterility testing	- 2
Incubation	
1. Preparation of lunar material	+ 1
2. Inoculation	+ 1
3. Incubation	+1 to +14
Backup studies	
1. Identification	+2 to +21
2. In vivo tests	+2 to +60

TABLE 3.2.2-VI.- LOGISTICS OF INCUBATOR SPACE REQUIRED FOR CONTROLLED-ENVIRONMENT ENCLOSURES AND TEST TUBES

Sample	Temperature, °C	Enclosures	Tubes
Prime	4	18	6
	24	18	6
	35	18	6
	55	18	6
	Total	72	24
Pooled	4	30	12
	24	30	12
	35	30	9
	55	30	9
	Total	120	42

TABLE 3.2.2-VII.- LOGISTICS OF MEDIA REQUIRED

Sample	Plates	Tubes	Plate controls	Tube controls
Prime sample				
Indigenous	12		12	
Blood agar	48		24	
Trypticase glucose yeast extract	48		24	
Thioglycollate broth	—	16	—	8
Total	108	16	60	8
Pooled sample				
Indigenous	12		12	
Blood agar	48		24	
Trypticase glucose yeast extract	48		24	
Thioglycollate broth		16	8	
Czapek Dox	48		24	
Sabouraud dextrose	48		24	
Soil extract	24		12	
Aquatic media	—	12	6	
Total	228	28	134	
Grand total	444	60	158	

(c) All Class III Cabinetry will be sterility tested every 24 hours. One sterile moist cotton swab will be used to sample the floor and walls of each cabinet section. The swab will be used to streak three trypticase glucose yeast extract agar plates. One plate each will be incubated at 25° (indigenous to cabinetry), 35°, and 55° C for 5 days. The plates will be observed every 24 hours for colony formation.

(2) Controlled-environment enclosures

(a) All enclosures will be certified for 100-percent isolation using Freon leak-detection apparatus.

(b) The methods used for certification are described in appendix A, OPV7S2.8.

(c) All enclosures will tolerate vacuums necessary to exchange atmospheres.

(d) The enclosures will be tested for pressure, vacuum, and heat lability.

(e) All enclosures will be sterilized with gas valves open in an atmosphere of ethylene oxide for a period of 16 hours or during formaldehyde sterilization of the Class III Cabinetry.

(3) Gases

(a) All gases used to meet atmospheric requirements within controlled-environment enclosures will be certified by the manufacturer.

(b) The individual cylinders will be certified to contain 10 percent CO_2 or prepurified nitrogen.

(c) Each cylinder of 10 percent CO_2 will be monitored for quality of gas when received.

(d) Each cylinder of prepurified nitrogen will be monitored for quality of gas when received.

(e) All gases will be sterilized by filtration before use in controlled-environment enclosures. The filter pore size will be 0.45μ.

(4) Incubators

Humidity will not be a factor because all cultures will be enclosed.

(a) Cabinet incubators will maintain required temperatures of 4°, 24°, 35°, 55° C and will have a sensitivity range of ±1.0° C.

(b) The refrigerated incubator cabinet (backmounted), located in room 1-123, will be used for 4° C incubation.

(c) The heated incubator (backmounted), located in room 1-123, will be used for 35° C incubation. The incubators will have a sensitivity of ±1.0° C.

(d) Independent heated incubators, located within room 1-123, Class III Cabinetry, will be used for 55° C incubation. The incubators will have a sensitivity of ±1.0° C.

(e) The Class III Cabinetry in room 1-123 will be used for 24° C incubation.

(f) All incubators will be equipped with temperature-recording devices, which will be observed periodically.

(5) Sterilization apparatus

All Class III Cabinetry autoclaves and ethylene oxide gas sterilizers will be tested according to procedures outlined in the LRL Health and Safety Manual V7S2, Quarantine Control Certification.

(6) Entry of equipment into Class III Cabinetry

(a) Autoclavable equipment (steam process)

1. The equipment will be placed in the autoclave in the Class III Cabinetry in room 1-123.

2. The equipment will be autoclaved at 121° C for 30 minutes.

3. The equipment will be removed through the inside door and passed to the rear arm of the Class III Cabinetry.

(b) Nonautoclavable equipment

1. Items which will not be passed through Class III autoclaves are given as follows.

   Items will be placed in open plastic bags in the carboxyclave in room 1-135-A. A sterilization cycle will be completed employing methods discussed in OMV4S9.

   Before removal from the carboxyclave, the bag will be sealed.

   Sterilized bagged equipment will be placed in Class III Cabinetry in Room 1-123 by passing through an open window.

   The cabinetry will subsequently be closed and sterilized with steam formaldehyde employing methods described in OPV7S2.

   After aeration of cabinetry for 48 hours by normal ventilation, the bagged equipment can be opened.

2. Nonautoclavable equipment which will be passed through Class III autoclaves (ethylene oxide gas process).

   Items will be sterilized by ethylene oxide gas in the autoclave attached to Class III Cabinetry in room 1-123 employing methods described in OMV4S9.

Items will be removed through the inside door and passed to the rear arm of the Class III Cabinetry.

3.2.2.4 Release of Sample

The recommendation of release of the lunar sample will be made according to the logic flow presented in figure 3.2.2-5.

3.2.2.5 Equipment used Inside Class III Cabinetry

1. Water bath, 1
2. Hotplate, 1
3. Controlled-environment enclosures, 192
4. Glass Petri plates, 60 × 15 mm, 600
5. Pipettes, disposable
   (a) 2 ml, 12
   (b) 5 ml, 12
   (c) Pasteur, 2 jars
   (d) Pasteur, calibrated, 6
6. Glass beakers
   (a) 600 ml, 5
   (b) 400 ml, 4
7. Medicine bottles, 16 oz, 4
8. Propipettes, 3
9. Squeegie bulbs, 6

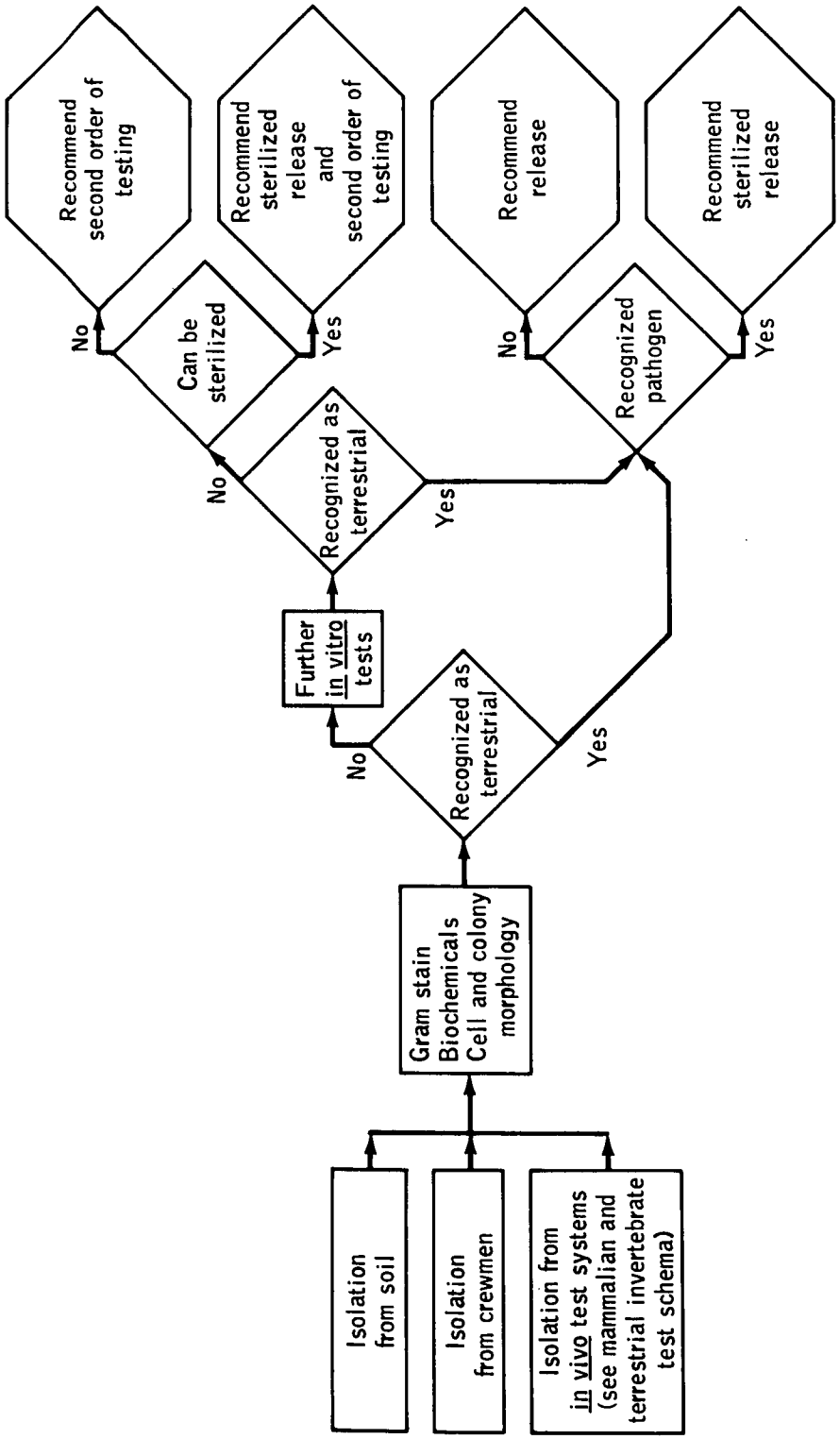


Figure 3.2.2-5.- Lunar-sample release flow chart for bacteriology and mycology.

10. Trays
    (a) 12 to 14 inches, 10
    (b) 10 to 12 inches, 3
11. Glass spreaders, 20
12. Inoculating loops, 2
13. Pens, 2
14. Pencils, 4
15. Diamond pens, 1
16. Wax pencils, 3
17. Microscope slides, frosted ends, 3 boxes
18. Forceps
    (a) Large, 2
    (b) Small, 2
19. Labels, 1 roll
20. Anaerobic indicator, 1 bottle
21. Fermentation tubes, 72
22. Sterile water, 32-oz bottles, 2
23. Squeeze bottles, 3
24. Hyperchlorite solution, 400 ml
25. Sponges, 3
26. Paper towels, 2 packs
27. Kimwipes, 2 boxes
28. Grippers, 3
29. Autoclave wrench, 1

30. Incubator tool, 1
31. Pliers, 1
32. Tygon tubing, 3/8", 4 ft
33. CO_2 gas cylinders, 3
34. N_2 gas cylinders, 3
35. Hot pad, 1
36. Immersion oil, 1/2 oz bottle, 1
37. Lens paper, 2 books
38. Gram-stain reagents, 1 set
39. Biochemical solutions, 1 set
40. Acid-fast stain reagents, 1 set
41. Sugars, 1 set
42. Staining tray, 1
43. Staining rack, 1
44. Discard pans, 2
45. Discard buckets, 2
46. Thermometers, 6

3.3 VIROLOGY

See section 1.3.1 for the lunar sample toxicity determination procedure which will precede other biological test procedures.

3.3.1 Analyses of Effects of Lunar Material on Tissue Cultures and Embryonated Eggs

See the addendum entitled, Analysis of Effects of Lunar Material on Fish Tissue Cultures, that follows section 3.3.3.

3.3.1.1 Objectives

The five objectives of the analysis protocol are: (1) to determine if infectious particles are present in lunar material by virtue of their ability to replicate in one or more cell cultures *in vitro* as well as in embryonated eggs *in vivo*, (2) to propagate the infectious agents isolated from lunar material to levels adequate for subsequent studies, (3) to investigate the biological, physiochemical, and antigenic properties of all infectious agents isolated from lunar samples, (4) to identify all agents isolated from lunar samples, and (5) to contain all agents capable of replication that are present in lunar samples.

3.3.1.2 Protocol

3.3.1.2.1 Tissue culture systems.-

I. Test design

a. Six types of cell culture will be exposed to prime and pooled lunar samples in an attempt to isolate agents capable of multiplication in tissue culture systems.

b. The lunar samples will be introduced into the host cell cultures as the supernatant from a 50-percent suspension of lunar material in tryptose phosphate broth containing 0.5-percent gelatin.

c. Any agent that is detected will be propagated, studied, and, if possible, identified.

II. Test method

a. Host — lunar samples for infectious particles. Six cell types are used as hosts to test

(1) Primary African green monkey kidney (GMK)

(2) Primary human embryonic kidney (HEK)

(3) Human embryonic lung, diploid (WI-38)

(4) Primary bovine embryonic kidney (BEK)

(5) Primary porcine embryonic kidney (PEK)

(6) Primary duck embryonic fibroblast (DEF)

b. Entry of host cultures into sterilized Class III Cabinetry

The containers with tissue cultures will be sprayed with disinfectant and passed through the autoclave into the Class III Cabinetry.

c. Sample requirement

(1) Infective dose

Each culture tube will be inoculated with the supernatant from a 50-percent suspension of lunar material (0.223 g of lunar material).

(2) Prime lunar sample requirement

(a) Sample — one

(b) Inocula — two

1 Supernatant from 50-percent suspension of soil

2 Supernatant from 50-percent suspension of sterile soil

(c) Media — two

1 Antibiotic

2 Antibiotic-free

(d) Cell culture containers — Eight screw-cap tubes/cell type

(e) Cell cultures for initial passage — 192

Eight cell cultures/cell type times six cell types times two inocula times two media times one sample

(f) Amount of prime sample — 42.82 g

One hundred and ninety-two cell cultures times 0.223 g of sample

(3) Pooled lunar sample

(a) Sample — one

(b) Inocula — two

1 Supernatant from 50-percent suspension of soil

2 Supernatant from 50-percent suspension of sterile soil

(c) Medium — one

Antibiotic

(d) Cell culture containers

Eight screwcap tubes/cell type

(e) Cell cultures for initial passage — 96

Eight cell cultures/cell type times six cell types times two inocula times one medium times one sample

(f) Amount of pooled sample — 21.41 g

Ninety-six cell cultures times 0.223 g of sample

d. Preparation of sample for inoculation

Diluent — tryptose phosphate broth containing 0.5-percent gelatin

(1) Prime sample

(a) From the Biopreparation Laboratory, obtain two 21.46 g of prime lunar soil samples

1 One dry-heat sterilized

2 One untreated

(b) Transfer each sample to a sterile 50-ml centrifuge tube graduated at 42.92 ml.

(c) Dilute to 42.92 ml.

(d) Centrifuge the suspended samples at 2500 rpm for 15 minutes.

(2) Pooled sample

(a) From the Biopreparation Laboratory, obtain two 24.08 g conventional lunar soil samples.

1 One dry-heat sterilized

2 One untreated

(b) Transfer each sample to a sterile 50-ml centrifuge tube graduated at 48.16 ml.

(c) Dilute to 48.16 ml.

(d) Centrifuge the suspended samples at 2500 rpm for 15 min.

e. Inoculation

(1) Volume

Each culture will be inoculated with 0.2 ml of the supernatant from the soil suspension.

(2) Procedure

Note

Sterile technique will be used throughout the following procedures.

(a) Stand the tissue culture tubes containing 1.0 ml of maintenance medium in a vertical position in a test tube support while the tubes are receiving the inoculum.

(b) Remove the caps from the group of culture tubes receiving the inoculum.

(c) Deliver the inoculum with a 2.0-ml pipette.

(d) Discard used pipettes into pans (with covers) for autoclaving.

(e) Close the inoculated culture tubes immediately with new sterile caps.

(f) Label each group of tubes with a coded number.

(g) Two hours after inoculation, examine the cultures and make any required adjustments of pH.

f. Incubation

(1) Temperature

(a) Incubate the DEF cell cultures at ambient temperature in a Class III Cabinetry. The culture tubes will be held in stationary racks.

(b) Incubate mammalian cell cultures at 35° C in a Class III incubator. The culture tubes will be held in stationary racks.

(2) Length of incubation (See appendix A)

(a) Incubate one-half of the cultures for 7 days. If no infective process is detected, make three blind subpassages (approximately 28 days).

(b) Incubate the other half of the cultures for 14 days for detection of slow-growing agents. If no infective process is detected, make two blind subpassages (approximately 42 days).

(3) Scheme

(a) Immediately subpassage all cell cultures showing degeneration within the first 24 hours after being inoculated with lunar material. Degeneration occurring within the first 24 hours is usually a result of toxicity of the inoculum and a viral infection. This effect may be diluted out by subpassage, thus allowing any infectious agent to replicate.

(b) Observe cell cultures daily for evidence of infection.

1 Examination will be made by direct microscopic observation with both low- and high-power objectives and will be carried out in the Class III Cabinetry.

2 Take 35-mm photographs of a random number of negative culture tubes at each observation of cell strains. Color photographs will be taken of all materials exhibiting abnormal changes indicative of the presence of viral agents.

(c) Feed the tissue culture, as required, by adding 1.0 ml of maintenance medium.

(d) For passage (see section 3.3.1.2.1, Part III), harvest the cell cultures by scraping the cells off the side of the tube with a rubber policeman and pool each group of cultures. Perform hemagglutination procedures on all pools. Inoculate the pooled material into homologous tissue cultures; the remaining inoculum will be stored frozen at -70° C. If cell cultures are not available for passage, the material will be stored frozen at -70° C until passage can be effected.

(e) The fate of cultures in final blind passage (section 3.3.1.2.1, part III; 0PV4S8.2.2.1.2H).

1 Use two inoculated screwcap culture tubes and two controls for the detection of inapparent infection by interference studies. Challenge the cultures with vesicular stomatitis virus on the 7th to 10th day of incubation. Discard the cultures at the conclusion of the test by autoclaving them out of the Class III Cabinetry.

2 Use two inoculated screwcap culture tubes and two controls for the detection of inapparent infection by plaque formation. Discard the cultures at the conclusion of the tests by autoclaving them out of the Class III Cabinetry.

3 Test two inoculated screwcap culture tubes and two control cultures for infection by hemadsorption with the following red blood cells: guinea pig, sheep, goose, monkey, rat, and human O. Utilize the red blood cells in sequence. Discard the cultures

at the conclusion of the tests by autoclaving them out of the Class III Cabinetry.

4 Harvest two inoculated screwcap culture tubes and two control cultures, pass the cultures out of the Class III Cabinetry, and store them at -70° C.

5 Use two inoculated Leighton tube cultures and two control Leighton tube cultures for histological examination. The Leighton tube cultures will be treated in the following manner:

a Completely fill the Leighton tubes with 10 percent formalin in absolute alcohol and close the tubes. Leave the tubes in the Class III Cabinetry for 24 hours.

b Pass the tubes containing alcohol-formalin to the outside of the Class III Cabinet through the dunk tank, and transfer the tubes to room 147 as specified in OPV7S2.13.

c Stain the cover slips with hematoxylin and eosin stain, and safranin stain. (See appendix B.)

6 Use one inoculated prescription bottle (16 oz) culture and one control prescription bottle (16 oz) culture for ultramicroscopic examination. The cultures will be treated in the following manner:

a Decant and discard the medium from each bottle.

b Wash the monolayer two times with Millonig's phosphate buffer.

c Add 5.0 ml of 3 percent buffered glutaraldehyde, remove the cell sheets with a rubber policeman, transfer the suspension into a centrifuge tube, and close with a rubber stopper. Leave the tubes in the cabinetry for 24 hours.

d Centrifuge the tubes containing the suspension at 2000 rpm for 30 minutes, pass the tubes out of the cabinetry through the dunk tank, and transfer them to room 147.

e Process the pellet for examination with the electron microscope by the procedures in appendix C.

g. Observations

(1) Direct microscopic examination of cell cultures

Morphological changes in cell cultures inoculated with lunar material will be recorded and photographed. Observations will be made for the following changes:

(a) Nonspecific degeneration

The cells may rapidly become rounded and detach from the glass surface.

(b) Cytopathic effect (CPE)

An infective process may produce characteristic changes in cell morphology. Alterations in morphology may be one of the following types:

1 The cells in the monolayer may become rounded and refractile in focal lesions that spread until the cell sheet has detached from the glass surface.

2 The cells may become rounded and very large and tend to aggregate in grape-like clusters.

3 The cells may become rounded and tend to have a chain-like configuration.

4 The cells may become rounded and tend to bunch up but will demonstrate little enlargement of individual cells. The cells may have a dull, rather than a refractile, appearance. Focal lesions within the cell sheet may be self-limiting in spread.

5 The cells may merge or lose their cytoplasmic borders and form one giant or syncytial cell. These cells may contain many nuclei which aggregate in the center of the large cells. These cells may also contain inclusion bodies.

6 The cells may develop a characteristic stellate or spindle shape.

7 The cells may have a characteristic foamy type of generation.

(c) Any change in appearance of the experimental cell cultures not present in the control cultures.

(2) Metabolic inhibition

Infection of cell cultures may be detected by failure of the phenol-red indicator of the maintenance medium to turn from red to yellow (the change from red to yellow occurs naturally as a result of cellular metabolism).

(3) Histological examination of cell cultures

Stained preparations of cell culture monolayers will be examined for the following cell changes:

(a) Presence of viral inclusions

(b) Presence of abnormal crystals or granules within the cells

(c) Changes in the staining properties of the cells

(d) Alteration of genetic material

1 Distortion of normal mitosis

2 Breaks, distortions, or fusions of chromosomes

(e) Separation of cells from each other

(f) Changes in the morphology of the cell

1 Pyknosis, fragmentation, or disintegration of the nucleus

2 Loss in intranuclear structure

a Loss of chromatin or aggregation of chromatin at nuclear border

b Disintegration of nucleolus

3 Production of vacuoles in cytoplasm

4 Change the size of the nucleus in relation to the cytoplasm

(4) Ultramicroscopic examination of cell cultures

Electron micrographs will be examined for the following changes in the ultrastructure of the cells.

(a) Loss of differentiation of the cells

(b) Presence of broken cell walls

(c) Presence or absence of fibrous material

(d) Change in the number and size of golgi bodies, rhibosomes, secretion granules, and mitochondria

(e) Presence of viruses, mycoplasma, bacteria, or fungi

(f) Aggregation of nuclear material at the nuclear membrane

h. Backup studies

(1) Titration of infectious particles

(a) Tissue culture 50-percent infective dose ($TCID_{50}$)

(b) Plaque assay

(2) Identification studies

(a) Plaque morphology

(b) Hemagglutination

(c) Hemagglutination inhibition

(d) Hemadsorption

(e) Hemadsorption inhibition

(f) Neutralization in cell culture

(g) Complement fixation

(h) Ether and/or chloroform sensitivity

(i) Acid lability

(j) Iododeoxyuridine inhibition

(k) Heat stability

(1) Effect on other hosts

1 Newborn and adult mice

2 Embryonated eggs

3 Newborn hamsters

i. Emergency plans

(1) Host failure

(a) Arrangements will be made with commercial suppliers to obtain a complete supply of seeded tubes and flasks of each cell type needed for the program. These cultures will be ready for immediate shipment by air express to replace any cell culture failures.

(b) A large supply of cells derived from freshly trypsinized organs will be available in the nitrogen freezer (monolayers grown from this source may require up to 10 days for development). These tissues will be from the same lot of materials employed as the primary host systems.

(c) Continuous cell types will be in culture in the laboratory at all times and will be available for preparation of the needed tubes and flasks (monolayers will require approximately 2 days to develop). In addition, a supply of continous cells will be kept in the nitrogen freezer.

The following cell cultures will be available for cross-utilization as required:

Primary	Backup
Primary HEK	Hep-2
Primary African GMK	Vero
WI-38	WI-38
Primary DEF	Primary chick embryonic fibroblast
Primary BEK	Bovine trachea
Primary PEK	PK_{15}

(2) Technician failure

Available personnel in the Virology and Tissue Culture Group will be cross-trained.

(3) Equipment failure

Duplicates of the following pieces of equipment will be available in the Support Laboratories and will be available for transfer behind the barrier in case of failure.

(a) Microscopes (upright and inverted)

(b) Water baths

(c) Magnetic stirrers

(d) Clinical centrifuge

(e) Camera

(f) Ample dry ice on hand to charge the freezers in the event of power failure.

(g) Auxiliary power supply for all electrical equipment employed in the Support and Sample Laboratories.

j. Exit of materials from Class III Cabinetry during mission

(1) Autoclavable material

(a) Place the material to be steam autoclaved in the cabinet autocalve as outlined in the Biological Safety Manual, Report Number MSC 00018.

(b) Remove the material through the outside door of the steam autoclave.

(2) Nonautoclavable material to be carboxyclaved

(a) Place materials to be sterilized in the cabinet autoclave and expose to ethylene oxide gas for a period of 16 hours.

(b) Remove the materials through the outside door of the autoclave.

III. Support effort

a. Materials required

(1) Cell culture requirements for initial passage

(a)	Prime lunar sample (eight screwcap tube cultures/cell type times six cell types times two media times two inocula)	192 cultures
(b)	Pooled lunar sample (eight screwcap tube cultures/cell type times six cell types times one media times two inocula)	96 cultures
(c)	Controls	
	Prime sample (eight screwcap tube cultures/ cell type times six cell types times two media)	96 cultures
	Pooled sample (eight screwcap tube cultures/ cell type times six cell types times one media)	48 cultures
(d)	Total cultures needed for isolation passage one, including overages:	
	Screwcap tube test	288 cultures
	Screwcap tube control	144 cultures
	Overage	18 cultures
	Total	450 cultures

(2) Cell culture requirements for blind passages

Three passages of 7-day incubated cultures and two passages of 14-day incubated cultures

(a) Prime lunar sample

Five blind passages (four screwcap tube cultures/cell type times six cell types times two media times two inocula times five passages)	480 cultures
Additional cultures for the final passages (four screwcap tube cultures/ cell type times six cell types times two media times two inocula)	96 cultures
Total for prime sample	576 cultures

(b) Pooled lunar sample

Five blind passages (four screwcap tube cultures/cell type times one media times two inocula times five passages)	240 cultures
Additional cultures for the final passages (four screwcap tube cultures/ cell type times six cell types times two inocula)	48 cultures
Total for pooled sample	288 cultures

(c) Controls

Prime sample (four screw-cap tube cultures/cell type times six cell types times two media times five passages)	240 cultures

Pooled sample	240 cultures
Additional cultures for the final passages	144 cultures
Total for blind passages	624 cultures

(d) Total screwcap tube cultures required for blind passages:

Prime sample	576 cultures
Pooled sample	288 cultures
Control	624 cultures
Overage	200 cultures
Total	1688 cultures

(e) In addition, the final blind passage will require:

1 Leighton tube cultures

Leighton tube test	72 cultures
Leighton tube control	72 cultures
Overage	56 cultures
Total	200 cultures

2 Prescription bottle cultures

Prescription bottle (16 oz) test	36 cultures
Prescription bottle (16 oz) control	36 cultures
Overage	28 cultures
Total	100 cultures

(3) Cell culture maintenance medium

The cell culture maintenance medium is 49.5 percent Eagle's Minimal Essential medium, 49.5 percent medium 199, and 1 percent fetal bovine serum. The antibiotic medium contains 100 units of penicillin, 100 µg of streptomycin, and 50 µg of mycostatin

(a) Medium required for initial passage

1.0 ml times 450 tube cultures	450 ml	
Overage	100 ml	
Medium required		550 ml

(b) Medium required for blind passages

1.0 ml times 1688 tube cultures, Leighton tubes plus 200	1888 ml	
Overage	400 ml	
Medium required		2288 ml
10 ml times 100 prescription bottle (16 oz) cultures	1000 ml	
Overage	300 ml	
Medium required		1300 ml
Total medium required		4138 ml

b. Quality control work

(1) Cell cultures

The cell cultures to be used will be prepared from materials which are certified by the supplier as free of adventitious agents. The cultures will be analyzed by personnel in the LRL prior to use for the presence of

agents which can be cultivated and identified, or for those agents present in a "masked" or "suppressed" state which can only be detected by electron microscopy.

(2) Cell culture medium

Maintenance medium will be made up in lots which will be checked for sterility in the following manner:

(a) Incubate approximately 6 percent (three bottles) of each lot of medium for 10 days at 37° C.

(b) Subculture the three bottles on the first and seventh day of incubation to the following media: PPLO agar, blood agar, thioglycollate broth, and Sabouraud's medium.

(c) Test media for toxicity, utilizing the HeLa S3 cloning efficiency test.

(d) After all tests have been found to be negative, release the medium for use. If the cultures are test positive, autoclave and discard the medium.

(3) Harvested cultures

Each pool of harvested cultures of each blind passage will be checked for bacterial, fungal, and mycoplasma contaminants in the following manner:

(a) Inoculate 0.1 ml of each pool of harvested cultures into the following media: thioglycollate broth, PPLO agar, Sabouraud's medium, and blood agar.

(b) Identify organisms detected in the above media by bacteriological and mycological methods.

IV. Time schedules

a. Preparation of cell culture maintenance media

All lots of medium to be used on cell cultures that will be inoculated with lunar material will be available 2 weeks before the lunar sample arrives. The lots of medium will be tested for sterility and toxicity in the manner described under quality control work, part III, b.

b. Cell cultures will be prepared from frozen stocks. Approximately 10 days will be required to obtain the cultures required for inoculation.

c. The maintenance media on all cell cultures will be changed immediately before the cultures are passed into the Class III Cabinetry.

d. The cell cultures will be inoculated with lunar samples as soon as the latter can be prepared for testing (day no. 1).

e. Cell cultures incubated for 7 days will be sub-passaged on day no. 8 (blind passage one).

f. Cell cultures incubated for 14 days will be passaged on day no. 15 (blind passage one).

V. Pretest preparation of test area

a. Entry of equipment into Class III Cabinetry

(1) Autoclavable equipment (steam process)

(a) Place the equipment in the autoclave in the Class III Cabinetry in room 1-107.

(b) Autoclave the equipment at 121° C for 30 minutes and 15 psi pressure.

(c) Remove the equipment through the inside door and pass it to the rear arm of the Class III Cabinetry.

(2) Nonautoclavable equipment

Equipment which will be passed through Class III autoclaves (ethylene oxide gas process)

(a) Sterilize items with ethylene oxide gas in the autoclave attached to Class III Cabinetry in room 1-107.

(b) Remove items through the inside door and pass to the rear arm of the Class III Cabinetry.

b. Standards to be met

(1) Class III Cabinetry

(a) Certify all Class III Cabinetry for 100-percent isolation.

(b) Sterilize all Class III Cabinetry and supply with sterile air.

(2) Sterilize all equipment and apparatus used within Class III Cabinetry.

Sterilization apparatus

(a) Test all cabinetry autoclaves for the ability to kill Bacillus stearothermophilus spores.

(b) Test all cabinetry ethylene oxide gas sterilizers for the ability to kill Bacillus subtilis spores.

(3) Equipment and apparatus

(a) Steam-sterilize all autoclavable equipment in the cabinet autoclave for 30 minutes at 121° C and 15 psi pressure.

(b) Sterilize nonautoclavable material with ethylene oxide gas or pass it through the 5000-ppm sodium hypochlorite dunk tank.

c. Methods to be employed

Class III Cabinetry

(1) Certify all Class III Cabinetry as biological barriers by using Freon leak detectors (OPV7S2.8).

(2) Sterilize all Class III Cabinetry with paraformaldehyde according to procedures outlined in OPV7S2.14.

(3) Sterility test all Class III Cabinetry by swabbing and inoculating trypticase glucose yeast extract agar (OPV4S12.2.3) and Chanock, Hayflick, and Barile's Diphasic Mycoplasma medium.

(4) Monitor cabinet air by employing trypticase glucose yeast extract agar plates and Chanock, Hayflick, and Barile's Mycoplasma agar plates in Reynier air samplers operated at 1 cfm (OMV4S14.6).

3.3.1.2.2 Embryonated eggs.-

I. Test design

Using 6- and 10-day-old embryonated eggs, three groups consisting of five eggs each will be inoculated with prime or pooled lunar material by one of four routes as follows:

a. Group one (10-day-old eggs) — Amniotic sac-allantoic sac

b. Group two (6-day-old eggs) — Yolk sac

c. Group three (10-day-old eggs) — Chorioallantoic membrane (CAM)

The lunar material will be introduced into the eggs as the supernatant from a 50-percent suspension in tryptose phosphate broth containing 0.5-percent gelatin.

The fluids and membranes will be harvested at the end of the incubation time and used for subpassage. Harvested fluids will be tested for the presence of

hemagglutinins, and membranes will be examined for pocks.

Three blind passages will be performed in an attempt to detect and cultivate infectious particles in lunar samples.

Any agent that is detected will be propagated and studied, and its identification will be attempted.

II. Test methods

a. Host

Embryonated eggs from white Leghorn chickens bred resistance-inducing-factor-free will be used (Kimber Farms, Fremont, California, will be the supplier).

b. Entry of embryonated eggs into Class III Cabinetry

(1) Immerse the eggs in a 1:256 solution of Vestal 1 Stroke Environ for 15 to 30 seconds.

(2) Place eggs in sterile racks and pass into cabinetry through autoclave.

c. Sample requirements

(1) Infective dose

Each egg will be inoculated with the supernatant from a 50-percent suspension of lunar material (0.223 g of lunar material).

(2) Prime lunar sample

(a) Samples — one

(b) Inocula — two

1 Supernatant from 50-percent suspension of soil

2 Supernatant from 50-percent suspension of sterile soil

(c) Eggs for the initial passage — 30

Five eggs/group times three groups times two inocula times one sample

(d) Amount of prime sample — 6.69 g

Thirty eggs times 0.223 g

(3) Pooled lunar sample

(a) Sample — one

(b) Inocula — two

1 Supernatant from 50-percent suspension of soil

2 Supernatant from 50-percent suspension of sterile soil

(c) Eggs for the initial passage — 30

Five eggs/group times three groups times two inocula times one sample

(d) Amount of pooled sample — 6.69 g

Thirty eggs times 0.223 g sample

d. Preparation of sample for inoculation

(1) Prime lunar sample

(a) Obtain two 3.345-g aliquots of prime sample from the Biopreparation Laboratory. One sample will be dry-heat sterilized and the other will be untreated.

(b) Transfer each sample to a sterile 10-ml screwcap tube graduated at 6.690 ml.

(c) Dilute each sample to volume (50-percent suspension) with tryptose phosphate broth containing 0.5-percent gelatin.

(d) Centrifuge the suspended samples at 2500 rpm for 15 minutes.

(2) Pooled lunar sample

(Same as prime lunar sample)

e. Inoculation

(1) Routes

(a) Amniotic sac-allantoic sac

(b) Yolk sac

(c) CAM

(2) Volume

Inoculate each embryonated egg with 0.2 ml of inoculum.

(3) Preparation of eggs (according to route)

(a) Amniotic sac-allantoic sac

1 Candle the embryonated eggs, and mark off the position of the embryo and the limit of the air sac with a pencil.

2 Punch a hole in the blunt end of the egg near the embryo (amniotic sac).

3 Punch the same egg in the center of the blunt end of the egg (allantoic sac).

(b) Yolk sac

Prepare eggs in the same manner as for amniotic sac inoculation.

(c) CAM

1 Prepare eggs in the same manner as described under amniotic sac except also cut a "window" in an area free

of blood vessels with a straight hand drill.

2 Drop the membrane under the "window" by applying negative pressure to the hole in the blunt end of the egg.

3 Remove the shell piece from the "window."

(4) Introduction of inoculum (according to route)

Inoculate the yolk sac samples first and then add the following amounts of antibiotics to the inoculum for inoculation of additional eggs by the amniotic-allantoic or CAM routes.

100 units of penicillin/ml of inoculum

100 µg of streptomycin/ml of inoculum

(a) Amniotic sac

1 Introduce the sample into the sac with a 1-ml sterile disposable syringe fitted with a 1-1/2-inch long, 20-gage needle.

2 Pass the needle through the hole in the blunt end of the egg and, with a quick motion, "hit" the amniotic sac with the needle (the embryo should jump).

3 Reclean the area of penetration at the blunt end of the egg and seal with adhesive sealing compound.

4 Place the needle and syringe into a disposal pan for autoclaving before disposal.

(b) Allantoic sac

Introduce the sample into the allantoic sac in the same manner as described under amniotic sac, except position the needle away from the embryo toward the shell.

(c) Yolk sac

Introduce the sample into the yolk sac in the same manner as described under amniotic sac, except position the needle directly toward the center of the egg.

(d) CAM

Using the "window," introduce the sample by dropping the inoculum onto the exposed membrane with a needle and syringe.

(e) Controls

Five eggs each will be inoculated with sterile diluent by the following routes:

1 Amniotic sac-allantoic sac

2 Yolk sac

3 CAM

f. Incubation

(1) Preinoculation

(a) Length of incubation (according to the route)

1 Yolk sac — 6 days

2 Amniotic sac — 10 days

3 Allantoic sac — 10 days

4 CAM — 10 days

(b) Conditions of incubation

Incubate the embryonated eggs with the blunt end up, at 38° C and at a 50- to 70-percent relative humidity environment. Turn the eggs every 4 hours.

(2) Postinoculation

(a) Length of incubation (according to route)

Incubate the eggs inoculated via the amniotic-allantoic and CAM routes routinely for 96 hours.

Incubate all eggs inoculated by the sac route for 6 days.

(b) Incubate the embryonated eggs with the blunt end up, at 36° C, and at ambient humidity. Do not rotate the eggs. Incubate eggs inoculated by the CAM route on their sides with the inoculated membrane upright.

(3) Scheme

(a) Nonspecific death of embryonated eggs

Discard embryonated eggs that die within the first 24 hours and do not count them in the final results (death occurring within the first 24 hours is usually a result of trauma or toxicity of the inoculum).

(b) Carry out each of the three blind passages in the same manner. The number of eggs inoculated in each passage will remain constant.

g. Harvest

(1) Methods (according to route)

(a) Allantoic and amniotic fluids

1 Place the egg in a support with the blunt end facing upward.

2 Clean the blunt end of the egg with a 1:256 solution of Vestal 1 Stroke Environ.

3 Break away the shell over the air sac, and display the shell membrane.

4 Aspirate the allantoic fluid with a sterile 5-ml serological pipette and a propipette.

5 Pool the allantoic fluids from the five eggs that received the same inoculum.

6 Remove the fluids from the amniotic sac with a 1-ml syringe fitted with a 26-gage needle. (Flush out dry amniotic sacs with 1 to 2 ml of the allantoic fluid harvest.

(b) Yolk sac

1 Prepare the egg in the same manner as described under "allantoic and amniotic fluids."

2 Loosen the CAM and decant the contents of the egg into a sterile Petri dish.

3 Detach the yolk sac from the embryo, rupture the sac, and allow it to drain.

4 Transfer the drained membranes to another sterile Petri dish and photograph.

(c) CAM

1 Clean the shell over the false air sac of the egg with a 1:256 solution of Vestal 1 Stroke Environ, and cut the shell away with sterile forceps and scissors, exposing the CAM.

2 Cut away the exposed portion of the CAM and place it in a sterile Petri dish. Examine and/or photograph, or place in a mortar and grind it with a pestle.

(2) Fate of harvested fluids and membranes

(a) Fluids (amniotic and allantoic)

1 Test each allantoic pool and amniotic pool with guinea pig and chick red blood cells for hemagglutinating particles.

2 Combine allantoic and amniotic pools for subpassage.

3 Store the remainder of each pool outside of the Class III Cabinetry at -70° C. The procedure for passing cultures to the -70° C freezer outside of the Class III Cabinet is described in this section, subpart k.

(b) Membranes (yolk sac and CAM)

1 Use one membrane of each inoculum (five eggs/inoculum) for histological examination.

Fix tissues in alcohol-formalin for 24 hours in the cabinetry and then remove the tissues to the Histology Laboratory employing procedures described in OPV7S2.13.

2 Use one membrane of each inoculum for ultramicroscopic examination.

a Wash the membranes two times with Millonig's phosphate solution.

b Transfer each membrane to a test tube and completely fill the tube with 3-percent buffered glutaraldehyde.

c Close the tube with a rubber stopper, and leave the tube in the cabinetry for 24 hours.

d Pass the tube out of the cabinetry through the dunk tank and transfer it to room 147.

e Prepare the membranes for examination by electron microscopy as described in appendix C.

3 Using a sterile mortar and pestle, the remaining three membranes of each inoculum will be respectively pooled and ground with sterile Alundum and used for subpassage and/or storage at -70° C. The procedure will be as follows:

a Grind the three membranes thoroughly with sterile Alundum in a mortar and pestle.

b Add 3 ml of tryptose phosphate broth with 0.5-percent gelatin and mix well.

c Sediment the suspension by centrifugation.

d Remove the supernatant and use for subpassage and storage.

h. Observations

(1) Perform the following gross observations and photography of the eggs within the Class III Cabinetry.

(a) Death of the embryo (no movement is detected when the egg is candled)

(b) Retraction of blood vessels (retraction indicates the embryo is dead or dying)

(2) Histological examination of membranes.

Examine fixed and strained membranes for the presence of intracellular viral inclusions as well as for pathological changes in the cells.

i. Backup studies

(1) Hemagglutination titer

(2) Effect on other systems

(a) Tissue culture

(b) Newborn and adult mice

j. Exit of materials from Class III Cabinetry during mission

(1) Autoclavable material

(a) Place the material to be steam autoclaved in the cabinet autoclave as outlined in the Biological Safety Manual, Report Number MSC 00018.

(b) Remove the material through the outside door of the steam autoclave.

(2) Nonautoclavable material to be carboxyclaved

(a) Place materials to be sterilized in the cabinet autoclave and expose to ethylene oxide gas for a period of 16 hours.

(b) Remove the material through the outside door of the autoclave.

(3) Procedures for passing harvested material to -70° C freezer outside of Class III Cabinetry

(a) Place the material to be stored at -70° C into double-stoppered Virtis vials.

(b) Pass the vials into the 5000-ppm sodium hypochlorite dunk tank for a sterilization time of 30 minutes.

(c) Put the vials into metal storage containers and seal the containers with silicon stoppers.

(d) Place the containers in the -70° C Revco freezer in room 1-135.

k. Emergency plans

(1) Host failure

(a) A backup supply of 100 eggs (10-day-old and 6-day-old) will be available in the Support Laboratory at the time of each passage.

(b) A backup supply of 100 eggs (10-day-old and 6-day-old) will be available from the supplier at the time of each passage.

(2) Technician failure

Available biological sciences personnel in the LRL will be cross-trained.

(3) Equipment failure

Duplicates of the following pieces of equipment will be available in the support laboratories and will be available for transfer behind the barrier in case of failure.

(a) Egg candler

(b) Clinical centrifuge

III. Support effort

a. Materials required

(1) Embryonated eggs needed for one passage

(a) Prime lunar sample

1 Ten-day-old embryonated eggs

Five eggs/group times two groups times two inocula times one sample = 20 eggs

2 Six-day-old embryonated eggs

Five eggs/group times one group times two inocula times one sample = 10 eggs

(b) Pooled lunar sample

1 Ten-day-old embryonated eggs

Five eggs/group times two groups times two inocula times one sample = 20 eggs

2 Six-day-old embryonated eggs

Five eggs/group times one group times two inocula times one sample = 10 eggs

(c) Control

1 Ten-day-old embryonated eggs

Five eggs/group times two routes = 10 eggs

2 Six-day-old embryonated eggs

Five eggs/group times one group/route = five eggs

(d) Total number of embryonated eggs needed for one blind passage, including overages

1 Ten-day-old embryonated eggs

Prime sample	30 eggs
Overage	30 eggs
Total (for prime sample)	60 eggs

Pooled sample	30 eggs
Overage	30 eggs
Total (for pooled sample)	60 eggs

2 Six-day-old embryonated eggs

Prime sample	15 eggs
Overage	15 eggs
Total (for prime sample)	30 eggs

Pooled sample	15 eggs
Overage	15 eggs
Total (for pooled sample)	30 eggs

(2) Embryonated eggs needed for three passages

(a) Ten-day-old eggs

1 Prime sample — three passages times 30 = 90 eggs

2 Pooled sample — three passages times 30 = 90 eggs

(b) Six-day-old eggs

1 Prime sample — three passages times 15 = 45 eggs

2 Pooled sample — three passages times 15 = 45 eggs

b. Quality control

(1) Microbiological status of the embryonated eggs will be determined by the supplier prior to shipment.

(2) Determination of the sterility of eggs

A representative sample (10 percent) of eggs will be tested for bacteria, fungi, and mycoplasma with the following media: blood agar, PPLO agar, thioglycollate broth, and Sabouraud's medium.

(3) Each pool of harvested fluids and membranes of each blind passage will be tested for bacteria, fungi, and mycoplasma in the following manner:

(a) Inoculate 0.1 ml of each pool into the following media: thioglycollate broth, blood agar, PPLO agar, and Sabouraud's medium.

(b) Organisms detected in the above media will be identified by bacteriological and mycological methods.

IV. Time schedules

Blind passages

a. Embryonated eggs inoculated by the amniotic-allantoic sac and CAM routes will be routinely incubated 4 days for each blind passage.

b. Embryonated eggs inoculated by the yolk sac route will be routinely incubated 6 days for each blind passage.

V. Emergency pool

An aliquot of each pool of harvested fluids or membranes will be stored at -70° C outside of the Class III Cabinetry in case of nonviral contamination or for future reference. The procedure for passing cultures to the -70° C freezer outside of Class III Cabinetry is described in section 3.3.1.2.2, part II, j.3.

VI. Pretest preparation of test area

Pretest preparation of test area will be the same as that described in section 3.3.1.2.1, part V.

3.3.2 Inoculation of Mycoplasma Media With Lunar Material

3.3.2.1. Objectives

The four objectives of the mycoplasma media inoculation protocol are: (1) to determine if organisms of a mycoplasma-like nature are present in lunar samples; (2) increase the number of any mycoplasma-like organisms isolated from lunar material to a level adequate for subsequent studies; (3) investigate the physical, chemical, cultural and morphological properties of any mycoplasma-like organisms detected and to identify them, if possible; (4) contain all particles capable of replication that are present in lunar samples.

3.3.2.2 Protocol

I. Test design

a. The medium developed by Chanock, Hayflick and Barile (ref. 1), which supports the growth of all known mycoplasma species (ref. 2), will be inoculated with lunar material. The medium will be used as an agar plate and in a diphasic form (an agar layer in a bottle overlaid with broth).

b. The lunar samples will be introduced in/on the culture medium as the supernatant from a 50-percent suspension of lunar material. The suspending medium will be the broth form of the medium.

c. An equal number of uninoculated diphasic culture bottles and agar plates will accompany the inoculated cultures during incubation, as controls.

d. The diphasic cultures will be incubated for 3 days and then subpassaged to fresh diphasic medium and agar plates. Agar plates will be incubated at 35° C in a high humidity environment (80 percent), under 5 percent CO_2 and 95 percent N_2 for 2 weeks and examined each day for the appearance of microcolonies.

e. Two blind passages will be performed in an attempt to cultivate any mycoplasma-like organisms in lunar material.

f. Any agents of a mycoplasma-like nature isolated from lunar samples will be propagated, studied, and classified, if possible.

II. Test methods

a. Sample requirement

(1) Infective dose

Each diphasic medium bottle and agar plate will be inoculated with the extract from 0.223 g of lunar material.

(2) Prime lunar sample requirement

(a) Sample — one

(b) Inoculum — one (supernatant)

(c) Media — two

1 pH 7.8 (penicillin and thallium acetate)

2 pH 6.3 (penicillin)

(d) Cultures — four

Two cultures (one diphasic bottle and one agar plate) times one inoculum times one sample times two media

(e) Amount of prime sample — 0.9 g

Four cultures times 0.223 g

(3) Pooled lunar sample

Same as prime lunar sample

b. Medium — Chanock, Hayflick, and Barile's (ref. 1 and 2) modification of the formula described by Edward (ref. 3), Morton, Smith, and Liberman (ref. 4, and see appendix D).

(1) The medium will be used with the following modifications:

(a) Use agamma horse serum.

(b) Add Eagle's vitamins (final concentration 1.0 percent), arginine (10 mM), and glutamine (10 mM).

(c) Add glucose (final concentration 0.2 percent).

(d) Add thallium acetate (1:1000 dilution) and penicillin (1000 units/ml).

(2) Entry of mycoplasma media into the Class III Laboratory.

(a) Prepare all media in the support laboratory.

(b) Pass media into Class III Cabinetry through the autoclave.

c. Preparation and inoculation of sample

(1) Obtain prime and conventional samples from the Biopreparation Laboratory.

(2) Dilute each sample (0.9 g) to 1.8 ml, and centrifuge the suspension.

(a) Inoculate 0.2 ml of the supernatant of each sample into the pH 7.8 medium for:

1 One agar plate

2 One diphasic culture bottle

(b) Inoculate 0.2 ml of the supernatant into the pH 6.3 medium for:

1 One agar plate

2 One diphasic culture bottle

d. Inoculation

(1) Procedure

(a) Diphasic culture medium

1 Stand the culture bottles in a vertical position in the rear arm of the Class III Cabinetry in room 1-107 while receiving the inoculum.

2 Remove the caps from the group of bottles to receive the inoculum. Discard the caps into a stainless-steel pan that is equipped with a cover for later steam autoclaving out of the Class III Cabinetry in room 1-107.

3 Deliver the inoculum into the culture bottles with a 1.0-ml serological pipette and propipette.

4 Discard used pipettes into a disposal pan for later steam autoclaving out of the cabinetry.

5 Close the inoculated culture bottles immediately with new sterile caps. Label with the sample and inoculum information and the date.

6 Mix the inoculated cultures by gentle shaking.

(b) Agar plates

1 Lift the lid of the Petri dish.

2 Deliver the inoculum onto the agar surface with a 1-ml serological pipette and propipette.

3 Replace the lid of the dish.

(2) Controls

Include an equal number of uninoculated diphasic culture bottles and agar plates together with the inoculated cultures during incubation.

e. Incubation

(1) Temperature

The inoculated cultures and controls will be incubated at 36° C.

(2) Environment

Incubate the agar plates in a high humidity (80 percent) environment under 5 percent CO_2 and 95 percent nitrogen.

(3) Location

Incubate the inoculated cultures and controls in a portable, humidified, gassed incubator in the rear arm of the Class III Cabinetry in room 1-107. Supply gas to the incubator from tanks positioned on the outside of the cabinet.

(4) Time

(a) Incubate the diphasic cultures for 3 days. Subpassage the broth phase to fresh bottles and agar plates. Two blind passages will be made if there is no evidence of growth.

(b) Freeze a portion of broth from each blind passage and store at -70° C outside the Class III Cabinetry in case of contingencies, contamination, or failure of an isolate to multiply in a later passage.

(c) Incubate the agar plates for 2 weeks. If no growth is observed, discard the plates by autoclaving them out of the Class III Cabinetry.

f. Observations

(1) Examine the inoculated medium each day for evidence of growth.

(a) Examine the broth phase of the diphasic cultures for turbidity and change in the pH of the medium (only slight turbidity is produced by growth of mycoplasma in broth).

(b) Examine the agar plates each day with the microscope mounted in the rear arm of the Class III Cabinetry in room 1-107. Use the 10X and 40X objectives when observing for the appearance of microcolonies which usually have the typical "fried egg" appearance.

(2) Compare all inoculated media to the control media.

(3) Record all operations carried out in the Class III Cabinetry in a log book.

(4) Take color slides of all positive agar plates.

g. Backup studies

(1) Identification studies

(a) Hemolysis employing guinea pig cells (appendix E, part I)

(b) Hemadsorption, employing guinea pig cells (appendix E, part II)

(c) Growth inhibition (appendix E, part III)

(d) Complement fixation (ref. 5)

(e) Gel-diffusion (appendix E, part IV)

(f) Indirect hemagglutination (appendix E, part V)

(g) Reaction of colonies with Dienes' stain (appendix B)

(h) Metabolic inhibition (appendix E, part VI)

(2) Any isolate will be compared with crew and spacecraft flora.

(3) Pathogenicity studies

Isolates not identified (or isolates not identifiable as originating from crewmembers or from the spacecraft) will be used to challenge germ-free mice.

h. Exit of materials from Class III Cabinetry during the mission

(1) Autoclavable material

(a) Place the material to be steam autoclaved into the cabinet autoclave for 30 minutes at 121° C and 15 psi pressure.

(b) Remove the material through the outside door of the steam autoclave.

(2) Nonautoclavable material to be carboxyclaved

(a) Place materials to be sterilized into the cabinet autoclave, and expose them to ethylene oxide gas for a period of 16 hours at ambient temperature.

(b) Pass the vial into the 5000-ppm sodium hypochlorite dunk tank for a period of 30 minutes.

(3) Procedures for passing cultures to -70° C freezer outside of the Class III Cabinetry

(a) Place material to be stored into double-stoppered virtis vials.

(b) Pass the vials into the 5000-ppm sodium hypochlorite dunk tank for a period of 30 minutes.

(c) Place the vials into metal storage containers and seal the containers with silicon stopper.

(d) Place the container inside the -70° C freezer located at the end of the bench in room 1-107, for storage.

i. Emergency plans

(1) Medium

(a) A 50-percent overage will be available within the Class III Cabinetry.

(b) A 100-percent overage will be available in the Sample Laboratory.

(2) Equipment failure

(a) The following equipment will be available within the Class III Cabinetry:

1 15- by 60-mm glass Petri dishes

2 4-oz screwcap prescription bottles

3 Propipettes

4 Pipettes

5 Pasteur pipettes

6 Marking pencils

7 Bottle caps

8 Light bulbs for microscopes

(b) The following equipment will be available in the Sample Laboratory:

1 Water bath

2 Microscopes

3 Portable incubator

4 Clinical centrifuge

5 Analytical balance

6 Vortex mixer

7 Freezer, -70° C or lower

(3) Technician failure

Available personnel will be cross-trained.

III. Support effort

a. Materials required

(1) Complete medium requirements for two blind passages including controls and a 150-percent overage are as follows:

Item	No.
Bottles of diphasic pH 7.8 culture medium (penicillin and thallium acetate)	60
Bottles of diphasic pH 6.3 culture medium (penicillin)	60
Agar plates, pH 7.8 (penicillin and thallium acetate)	60
Agar plates, pH 6.3 (penicillin)	60

(2) Components of medium

(a) Reconstituted PPLO base

Item	Amount
PPLO broth base	400 ml
PPLO agar base	1200 ml

(b) A supply of dehydrated PPLO agar base and broth base will be stored in air-tight bottles in the Support Laboratory.

(c) A supply of two 500-ml batches of vitamin-yeast extract will be prepared, filter

sterilized, and stored at -20° C in the Support Laboratory.

(d) A supply of two 500-ml lots of agamma horse serum will be stored at -20° C in the Support Laboratory.

(3) Equipment

(a) Large equipment

Item	No.
Freezer (-70° C) or lower	1
Microscopes	
Portable incubator	2
Vortex mixer	2

(b) Small equipment

Item	No.
Sterile, 4-oz prescription bottles	130
Sterile 1.0 ml serological pipettes	50
13-ml centrifuge tubes	5
Contamination pans (with covers)	4

b. Quality control work

(1) Medium

(a) Chanock, Hayflick and Barile's medium, prepared in this laboratory, will be tested for its ability to support the growth of the following mycoplasma strains:

1 M. pneumoniae (Eaton's agent)

2 T strains

(b) All complete medium will be tested for sterility by incubation at 36° C for 18 hours in the Class III Cabinetry.

(c) Each lot of agamma horse serum and vitamin-yeast extract will be tested for sterility 2 weeks before receipt of the sample.

IV. Time schedules

Activity	Time in reference to receipt of sample
a. Class III Cabinetry preparation	
(1) Sterilization with steam formaldehyde	-10 days
(2) Sterility testing	-8 days
b. Medium preparation	
(1) Preparation and sterilization of vitamin-yeast extract	-14 days
(2) Initiation of sterility testing of vitamin-yeast extract and horse serum	-14 days
(3) Preparation of complete medium	-1 day
(4) Initiation of sterility testing of complete medium	-1 day
c. Inoculation	
(1) Preparation of lunar material	+1 day
(2) Inoculation	+1 day
(3) Incubation	+1 to +21 days

V. Pretest preparation of test area

a. Entry of equipment into Class III Cabinetry

(1) Autoclavable equipment (steam process)

(a) Place the equipment in the autoclave in the Class III Cabinetry in room 1-107.

(b) Autoclave the equipment at 121° C for 60 minutes at 15 psi pressure.

(c) Remove the equipment through the inside door and pass it to the rear arm of the Class III Cabinetry.

(2) Nonautoclavable equipment

Equipment which will be passed through Class III autoclaves (ethylene oxide gas process)

(a) Sterilize items with ethylene oxide gas in the autoclave attached to the Class III Cabinetry in room 1-107.

(b) Remove items through the inside door and pass to the rear arm of the Class III Cabinetry.

b. Standards to be met

(1) Class III Cabinetry

(a) Certify all Class III Cabinetry for 100-percent isolation.

(b) Sterilize all Class III Cabinetry, and supply with sterile air.

(2) Sterilize all equipment and apparatus used within Class III Cabinetry.

(3) Sterilization apparatus

(a) Test all cabinetry autoclaves for the ability to kill Bacillus stearothermophilus spores.

(b) Test all cabinetry ethylene oxide gas sterilizers for the ability to kill Bacillus subtilis spores.

(4) Equipment and apparatus

(a) Steam-sterilize all autoclavable equipment in the cabinet autoclave for 30 minutes at 121° C and 15 psi pressure.

(b) Sterilize nonautoclavable material with ethylene oxide gas or pass through the 5000-ppm sodium hypochlorite dunk tank.

c. Methods to be employed

Class III Cabinetry

(1) Certify all Class III Cabinetry as biological barriers by using Freon leak detectors (OPV7S2.8; sec.3.1).

(2) Sterilize all Class III Cabinetry with paraformaldehyde in accordance with procedures outlined in OPV7S3.16.

(3) Sterility test all Class III Cabinetry by swabbing the inoculating trypticase glucose yeast extract agar (OPV4S12.2.3) and Chanock, Hayflick and Barile's Diphasic Mycoplasma medium.

(4) Monitor cabinet air by employing trypticase glucose yeast extract agar plates and Chanock, Hayflick and Barile's Mycoplasma agar plates in Reynier air samplers operated at 1 cfm (OMV4S14.6).

3.3.3 Characterization of Viral and Mycoplasma Flora of Apollo Crewmembers

3.3.3.1 Objectives

To isolate, characterize, and identify the mycoplasma and viral flora of Apollo crewmembers.

3.3.3.2.1 Protocol

Tissue cultures.-

I. Test design

a. Specimens will be analyzed for the presence of infectious viral agents by inoculating three cell types, embryonated eggs, and suckling mice.

b. Specimens will be tested for mycoplasma organisms by inoculating the medium developed by Chanock, Hayflick and Barile. This medium will be employed both as an agar plate and in a diphasic form.

II. Test method

a. Isolation systems (table 3.3.3-I)

(1) The specimens obtained from Apollo crewmembers will be employed to inoculate susceptible host systems. The following hosts will be employed in the analyses of one or more specimens:

(a) HEK

(b) WI-38

(c) GMK

(d) Embryonated eggs

(e) Suckling mice

(f) Mycoplasma medium, pH 7.8 and pH 6.3

TABLE 3.3.3-I.- CREW VIROLOGY INOCULATION SCHEMA

Host system	Whole blood	Specimen		Pharyngeal swab and gargle
		Urine	Feces	
Hayflick's medium	X	X	X	X
Embryonated eggs	X			X
Suckling mice	X		X	
HEK		X[a]	X[a]	X[a]
GMK			X[a]	X[b]
WI-38		X[a]		X[b]

[a]Incubated at 35° C.

[b]Incubated at 33° C.

(2) The following host tissue cultures (heterploid or diploid) will be available on a standby basis to back up the tissue culture host systems:

(a) Human epidermoid carcinoma of the larynx (H.Ep. no. 2)

(b) Monkey kidney, african green (vero)

(c) WI-38

(3) The crew specimens will be introduced into the host cell cultures following antibiotic pretreatment.

b. Entry of host culture vessels into sterilized Class III Cabinetry for postflight analyses

The containers with tissue cultures will be sprayed with disinfectant and passed into the Class III Cabinetry through the autoclave.

c. Specimen requirement

(1) The following specimens will be obtained from Apollo crewmembers:

(a) Feces

(b) Urine

(c) Pharyngeal swab

(d) Blood

(2) These specimens will be used to inoculate one or more host systems as shown in table 3.3.3.I.

d. Cell culture requirements for the isolation (initial) passage

Feces (Eight tubes/cell line times two cell lines times three men) — 48 tubes

Urine (Eight tubes/cell line times two cell lines times three men) — 48 tubes

Pharyngeal swab (Eight tubes/cell line times three cell lines times three men) —	72 tubes
Control (Eight tubes/cell culture times three cell lines) —	24 tubes
Total tubes required	192 tubes

e. Sample preparation

(1) Tissue cultures and mice

(a) Feces

1 Prepare a 10-percent suspension by placing 1.0 g of stool into a sterile 25-ml Erlenmeyer flask containing glass beads and add 9 ml of tryptose phosphate broth with 0.5 percent gelatin.

2 Shake the flask until the specimen is in suspension.

3 Transfer the suspension to a sterile centrifuge tube and sediment at 2500 rpm for 30 minutes. If required, higher centrifugal speeds will be employed.

4 Transfer the clarified supernatant to a sterile tube containing in a 1-ml volume, 100 000 units of penicillin, 100 000 µg of streptomycin, 50 µg of Fungizone, and 3 mgm of trypsin inhibitor.

5 Incubate the antibiotic-treated specimen at ambient temperature for 1 hour; mix occasionally.

6 Use an aliquot of 0.20 ml to inoculate each of eight cell containers of two cell types. Inject one litter (at least six mice) with an

aliquot of 0.025 ml via the intraperitoneal and intracerebral route.

(b) Urine

1 Bring a 9.0-ml sample of midstream urine to neutrality with Tris (hydroxymethyl) aminomethane buffer.

2 Add 1 ml of antibiotics containing 10 000 units of penicillin, 10 000 µg of streptomycin, and 50 µg of Fungizone.

3 Incubate the specimen at 4° C temperature for 1 hour; shake occasionally.

4 Use an aliquot of 0.20 ml to inoculate each of eight cell culture containers of two cell types.

(c) Pharyngeal swab

1 Immediately after swabbing the tonsil or pillars of the fauces and the posterior pharyngeal vault, immerse the swab in 6.0 ml of tryptose phosphate broth containing 0.5 percent gelatin.

2 Rinse the swab well in the collection medium and press the fluid out against the wall of the tube. Remove 1.0 ml and store at -10° C.

3 Add 50 ml of throat gargle and 1.0 ml of antibiotics containing 10 000 units of penicillin, 10 000 µg of streptomycin, and 50 µg of Fungizone.

4 Incubate the specimen at 4° C temperature for 1 hour.

5 Use an aliquot of 0.20 ml to inoculate each of eight cell containers of three cell types.

(d) Blood

1 Draw a 2.0-ml sample of blood, under sterile conditions, into 0.25 ml of 1:2000 dilution heparin.

2 Employ this specimen, without further treatment, to inoculate one litter (at least six mice) via the intracerebral route with 0.025 ml of sample.

(2) Eggs

Remove aliquots of the following specimens prior to antibiotic treatment as described for tissue cultures.

(a) Pharyngeal swab

Treat this specimen as described for tissue cultures, except that Fungizone will not be employed.

(b) Blood

Use an aliquot (with no antibiotic treatment) of 0.1 ml per egg to inoculate 6-day-old eggs via the yolk sac route.

(3) Mycoplasma

Remove aliquots of all specimens to be inoculated for mycoplasma isolations prior to antibiotic treatment as described for tissue cultures. Treat all specimens with 10 000 µg/ml of Staphcillin and 10 000 units/ml of penicillin at room temperature for 1 hour.

f. Virus isolation

(1) Inoculation

Inoculate each cell culture tube containing maintenance medium with 0.2 ml of inoculum; controls will receive tryptose phosphate broth containing antibiotics.

(2) Tissue culture medium

The maintenance medium described in section 3.3.1.2.1, part III, A3, with 100 units of penicillin, 100 µg of streptomycin, and 50 µg of mycostatin, is used.

(3) Incubation temperature

(a) Incubate the HEK and GMK cell cultures inoculated with feces and the WI-38 cell cultures inoculated with urine at 35° C on a roller drum. Incubate the HEK cell cultures inoculated with urine at 35° C in stationary racks.

(b) Incubate the GMK and WI-38 cell cultures inoculated with a pharyngeal specimen at 33° C on a roller drum. Incubate the HEK cell cultures inoculated with the pharyngeal specimen at 35° C on a roller drum.

(c) Incubate embryonated eggs at 36° C and elevated relative humidity

(d) Incubate mycoplasma isolation plates at 36° C in a humidified atmosphere of 5 percent carbon dioxide in 95 percent nitrogen.

(e) Maintain suckling mice under ambient conditions.

(4) Length of incubation

(a) Incubate one-half of the cultures for 7 days. If no infective process is detected, make three blind subpassages (approximately 28 days).

(b) Incubate the other half of the cultures for 14 days for detection of slow-growing agents. If no infective process is detected, make two blind subpassages (approximately 42 days).

(c) Prior to the passage of the cell cultures, test them for inapparent infections by performing hemadsorption and hemagglutination assays.

g. Detection of infection (see section 3.3.1.2.1, part II, f.3)

(1) Immediately subpassage all cell cultures showing degeneration within the first 24 hours after being inoculated with crew specimens. Degeneration occurring within the first 24 hours is usually a result of toxicity of the inoculum, and not of the viral infection. This rapid nonagent-induced degeneration may be diluted out by subpassage.

(2) Observe the cell cultures daily by direct microscopic observation for evidence of infection. Take photographs of random negative cultures from each cell system. Take additional photographs of all cell systems exhibiting abnormal changes indicative of the presence of viral agents.

(3) Feed the tissue cultures as required by adding 1.0 ml of maintenance medium.

h. Blind passage

(1) For passage, harvest cell cultures by scraping the cells off the side of the tube with a rubber policeman and pool each group of cultures.

(2) Inoculate the pooled material into homologous tissue cultures. Freeze and store the remaining material at -70° C. If cell cultures are not available for passage, the material will be stored frozen until passage can be effected.

(3) Harvest uninoculated control cell cultures and subpassage together with inoculated cultures of the same set.

(4) The number of cultures needed for all but final blind passage is as follows:

(a) Inoculated

Eight screwcap tube cultures/cell type/inoculum

(b) Control

Eight screwcap tube cultures/cell type/inoculum

(5) The number of cultures needed for the final blind passage is as follows:

(a) Inoculated

Eight screwcap tube cultures, two Leighton tubes, one 8-oz prescription bottle/cell type/inoculum

(b) Control

Eight screwcap tube cultures, two Leighton tubes, one 8-oz prescription bottle/cell type/inoculum

(6) Cytopathic effect (CPE) negative cultures from passages other than the final passage

Following 7 or 14 days of incubation, the cell cultures will be treated in the following manner:

(a) Assay for inapparent infections, employing the hemadsorption and hemagglutional reactions with guinea pig red blood cells.

(b) Pool identical cultures and use for reinoculation of the same number of tubes used in the original inoculation.

(c) Store an aliquot of each pool at -70° C.

(d) Check the sterility of each pool with the following media: blood agar, thioglycollate broth, Sabouraud's agar, and PPLO agar.

Deliver 0.1 ml of each pool onto each medium and incubate these under required conditions. Examine the cultures daily for growth.

(7) The fate of CPE negative cultures in final blind passage

(a) Use two inoculated screwcap culture tubes and two controls for the detection of inapparent infection by interference studies. Challenge these cultures with vesicular stomatitis virus on the 7th to 10th day of incubation; discard the cultures at the conclusion of the test by autoclaving them out of the Class III Cabinetry.

(b) Use two inoculated screwcap culture tubes and two controls for the detection of inapparent infection by plaque formation. At inoculation, remove the fluid medium from the tubes and wash the monolayers with maintenance medium. Inoculate the tubes, and, following a 2-hour adsorption period at ambient temperature, feed the monolayer with 1.0 ml maintenance medium containing 0.8 percent ion agar no. 2 and 1.0-mg/ml iodonitro-tetrazolium violet. Examine monolayers daily for evidence of plaques. Discard the cultures at the conclusion of the tests by autoclaving out of the Class III Cabinetry.

(c) Test two inoculated screwcap culture tubes and two control cultures for infection by hemadsorption with the following cells: sheep, guinea pig, goose, monkey, rat, and human O. Utilize the cells in sequence. Discard the cultures at the conclusion

of the tests by autoclaving out of the Class III Cabinetry.

(d) Harvest two inoculated screwcap culture tubes and two control cultures. Pass the cultures out of the Class III Cabinetry, and store them at -70° C.

(e) Use two inoculated Leighton tube cultures and two control Leighton tube cultures for histological examination.

The Leighton tube cultures will be treated in the following manner:

1 Completely fill the Leighton tubes with 10-percent formalin in absolute alcohol, and close the tubes. Leave the tubes in the Class III Cabinetry for 24 hours.

2 Pass the tubes containing alcohol-formalin of the Class III Cabinetry through the dunk tank and transfer them to room 147 where they will be examined as specified in OPV3S3.2.1.2, G3.

3 The cover slips will be stained with hematoxylin and eosin stain, and safranin stain. (See appendix 6B.)

(f) One inoculated prescription bottle (16 oz) culture and one control prescription bottle (16 oz) culture will be used for ultramicroscopic examination.

The cultures will be treated in the following manner:

1 Decant and discard the medium from each bottle.

2 Wash the monolayer two times with Millonig's phosphate buffer.

3 Add 5.0 ml of 3 percent buffered glutaraldehyde, remove the cell sheets with a rubber policeman, transfer the suspension into centrifuge tubes, and close each tube with a rubber stopper. Leave the tubes in the cabinetry for 24 hours.

4 Centrifuge the suspension at 2000 rpm for 30 minutes.

5 Pass the tubes out of the cabinetry through the dunk tank.

6 Transfer the tubes to room 147.

7 Process the pellet for examination with the electron microscope as described in appendix C.

(8) CPE positive cultures

(a) Harvest tubes which show extensive cytopathic degeneration (2 to 3+).

(b) Pool identical tubes showing CPE, titer in the same host system, and use for identification studies.

(c) Attempt a tentative identification on the basis of CPE type and the host system in which the agent was observed.

(d) Select a series of tests of identify this agent.

(e) Check the sterility of the pool with the following media: blood agar, thioglycollate broth, Sabouraud's medium, and PPLO agar.

(f) Store an aliquot of the pool at -70° C.

i. Identification studies

(1) Cell cultures with CPE, test of choice:

(a) Enteroviruses or rhinoviruses

1 Acid sensitivity

2 Neutralization test (serum pool) using cell monolayers in tubes.

(b) REO viruses

Hemagglutination inhibition

(c) Adenoviruses

Hemagglutination inhibition (monkey and rat cells)

(d) Herpes simplex v.

Neutralization test

(e) Respiratory syncytial virus

Complement fixation

(f) Measles virus

Neutralization

(2) Cell cultures with CPE not identifiable by the above tests:

(a) Arboviruses

Hemagglutination inhibition

(b) Herpes zoster-varicella

Complement fixation

(3) Cell cultures that hemadsorb, test of choice:

Hemadsorption inhibition

(4) Backup studies for virus identification

(a) General techniques

1 Determination of $TCID_{50}$

2 Plaque assay

(b) Enteroviruses

1 $MgCl_2$ heat stability

2 Ether treatment

3 Inoculation of newborn mice

4 Hemagglutination inhibition

(c) REO viruses

Complement fixation

(d) Adenoviruses

1 Complement fixation

2 Neutralization

(e) Herpes simplex v.

Pock formation on CAM

(f) Respiratory syncytial virus

Neutralization

(g) Measles

Hemadsorption with GMC

(h) Myxoviruses

Hemagglutination inhibition

j. Observations

(1) Direct microscopic examination of cell cultures

The following changes may be seen and photographed in cell cultures.

(a) Nonspecific degeneration

The cells may rapidly become rounded and detach from the glass surface.

(b) Cytopathic effect

An infective process may produce characteristic changes in cell morphology. Alterations in morphology may be one of the following types:

1 The cells may become rounded and refractile in focal lesions in the monolayer that spreads until the cell sheet has detached from the glass surface.

2 The cells may become rounded and very large and tend to aggregate in grape-like clusters.

3 The cells may become rounded and tend to have a chain-like configuration.

4 The cells may become rounded and tend to bunch up but will demonstrate little enlargement of individual cells. The cells may have a dull, rather than a refractile, appearance. Focal lesions within the cell sheet may be self-limiting in spread.

5 The cells may merge or lose their cytoplasmic borders and form one giant or syncytial cell. These cells may contain many nuclei which aggregate in the center of the large cells. These cells may also contain inclusion bodies.

6 The cells may develop a characteristic stellate or spindle shape.

7 The cells may have a characteristic "foamy" type of degeneration.

(c) Any change in the appearance of the experimental cell cultures that is not present in the control cultures.

(2) Metabolic inhibition

Infection of cell cultures may be detected by failure of the phenol-red indicator of the maintenance medium to turn from red to yellow (the change from red to yellow occurs naturally as a result of cellular metabolism).

(3) Histological examination of cell cultures

Stained preparations of cell culture monolayers will be examined for the following cell changes:

(a) Presence of viral inclusions

(b) Presence of abnormal crystals or granules within the cells

(c) Changes in the straining properties of the cells

(d) Alteration of genetic material

1 Distortion of normal mitosis

2 Breaks, distortions, or fusions of chromosomes

(e) Separation of cells from each other

(f) Changes in the morphology of the cell

1 Pyknosis, a fragmentation, or disintegration of the nucleus

2 Loss in intranuclear structure

a Loss of chromatin or aggregation of chromatin at nuclear border

b Disintegration of nucleolus

3 Production of vacuoles in cytoplasm

4 Change in the size of the nucleus in relation to the cytoplasm

(4) Ultramicroscopic examination of cell cultures

Electron micrographs will be examined for the following changes in the ultra structure of the cells.

(a) Loss of differentiation of the cells

(b) Presence of broken cell walls

(c) Presence or absence of fibrous material

(d) Change in the number and size of golgi bodies, rhibosomes, secretion granules, and mitochondria

(e) Presence of viruses, mycoplasma, bacteria, or fungi

(f) Aggregation of nuclear material at nuclear membrane

k. Backup studies

(1) Titration of infectious particles

(a) $TCID_{50}$

(b) Plaque assay

(2) Identification studies

(a) Plaque morphology

(b) Hemagglutination

(c) Hemagglutination inhibition

(d) Hemadsorption

(e) Hemadsorption inhibition

(f) Neutralization in cell culture

(g) Complement fixation

(h) Ether and/or chloroform sensitivity

(i) Acid lability

(j) Iododeoxyuridine inhibition

(k) Heat stability

(l) Effect on other hosts

1 Newborn and adult mice

2 Embryonated eggs

3 Newborn hamsters

1. Emergency plans

(1) Host failure

(a) Arrangements will be made with a commercial supplier to obtain a complete supply of seeded tubes and flasks of each cell type needed for the program. These cultures will be available for immediate shipment by air express if the in-house source is unavailable.

(b) A large supply of cells derived from freshly trypsinized organs will be on hand in the nitrogen freezer. Monolayers grown from this source will require 5 to 10 days for full development.

(c) Continuous cell types will be cultured in the laboratory at all times and will be available for preparation of the needed tubes and flasks. Monolayers grown from this source will require approximately 2 days to develop. In addition, a supply of these continuous

cell types will be kept in the nitrogen freezer.

The following cell cultures will be available for cross utilization as required:

Primary	Backup
Primary HEK	Hep-2
Primary African GMK	Vero
WI-38	WI-38

(2) Technician failure

Available personnel in the LRL Biological Sciences Section will be cross-trained.

(3) Equipment failure

The following materials will be available in the Support Laboratories for transfer behind the biological barrier in case of a failure.

(a) Magnetic stirrers

(b) Vortex mixers

(c) Clinical centrifuge

(d) Ample dry ice to maintain the freezers in event of a power failure

(e) Microscopes (upright and inverted)

(f) Water baths

(g) Camera

(h) Auxiliary power supply for all electrical equipment employed in the Support and Sample Laboratories

m. Exit of materials from Class III Cabinetry during a mission

(1) Autoclavable material

(a) Place the material to be steam autoclaved into the cabinet autoclave as outlined in the Biological Safety Manual, Report Number MSC 00018.

(b) Remove this material through the outside door of the autoclave.

(2) Nonautoclavable material

(a) Place materials to be sterilized into the cabinet autoclave and expose to ethylene oxide gas for a period of 16 hours at ambient temperature.

(b) Remove this material through the outside door of the autoclave.

(3) Procedure for passing biological samples to the freezer outside of the Class III Cabinetry.

(a) Place material to be stored at -70° C into double-stoppered Virtis vials.

(b) Immerse these containers in the dunk tank containing 5000-ppm available chlorine for 30 minutes.

(c) Place the vials into metal storage containers and seal the containers with silicon stoppers.

(d) Place the containers in the -70° C Revco freezer in room 1-135.

III. Support effort

a. Materials required, isolation (initial) passage

(1) Cell culture requirements

(a)	HEK or Hep-2	
	Inoculated (Eight tubes times three specimens times three astronauts)	72 tubes
	Control (Eight tubes)	8 tubes
	Total	80 tubes
(b)	GMK or Vero	
	Inoculated (Eight tubes times one specimen times three astronauts)	24 tubes
	Control (Eight tubes)	8 tubes
	Total	32 tubes
(c)	WI-28	
	Inoculated (Eight tubes times two specimens times three astronauts)	48 tubes
	Control (eight tubes)	8 tubes
	Total	56 tubes
(d)	Culture requirements for isolation passage (sum of (a), (b), and (c))	168 tubes
	Overage for isolation passage (Four tubes/cell culture/specimen/man)	72 tubes
	Total culture requirements	240 tubes
(2)	Cell culture maintenance medium	
	1.0 ml/culture tube times 240 tubes	240 ml
	Overage	160 ml
	Total medium required	400 ml

b. Materials required/blind subpassage 7-day or 14-day sequence

(1) Cell culture requirements

HEK or Hep-2	40 tubes
GMK or Vero	28 tubes
WI-38	28 tubes
Requirements for subpassage	96 tubes
Overage	44 tubes
Total	140 tubes

(2) Cell culture maintenance medium

1.0 ml/culture tube times 132	132 ml
Overage	68 ml
Total	200 ml

c. Materials required/final blind passage 7-day or 14-day sequence

(1) Cell culture requirements including overage

(a) HEK or Hep-2

1 Screwcap tubes	120
2 Leighton tubes	27
3 Prescription bottles (8 oz)	14

(b) GMK or Vero

1 Screwcap tubes	80
2 Leighton tubes	18

3	Prescription bottles (8 oz)	10
(c)	WI-38	
1	Screwcap tubes	80
2	Leighton tubes	18
3	Prescription bottles (8 oz)	10
(d)	Total screwcap tubes	280
	Total Leighton tubes	63
	Total prescription bottles	34

(2) Cell culture maintenance medium

1.0 ml/screwcap tube times 264 tubes plus overage	400 ml
2.0 ml/Leighton tubes times 54 tubes plus overage	150 ml
10.0 ml/8-oz prescription bottle times 32 bottles plus overage	400 ml
Total	950 ml

IV. Quality control work

a. Cell cultures

The cell cultures to be used will be prepared from materials which are certified by the supplier as free of adventitious agents. The cultures will be analyzed by personnel in the LRL prior to use for the presence of agents which can be cultivated and identified, or for those agents present in a "masked" or "suppressed" state which can only be detected by electron microscopy.

b. Cell culture medium

Maintenance medium will be made up in lots which will be checked for sterility in the following manner:

(1) Incubate approximately 6 percent (three bottles) of each lot of medium for 10 days at 37° C and 15 psi pressure.

(2) Subculture the three bottles on the first and seventh day of incubation to the following media: PPLO agar, thioglycollate broth, Sabouraud's medium, and blood agar. Test media for toxicity utilizing the HeLa S3 cloning efficiency test.

(3) After all tests have been found to be negative, release the medium for use. Contaminated or toxic media will be autoclaved and discarded. Acceptable medium will be stored at 40° C. Glutamine will be added prior to use.

c. Harvested cultures

Each pool of harvested cultures of each blind passage will be checked for bacterial, fungal, and mycoplasma contaminants in the following manner:

(1) Inoculate 0.1 ml of each pool of harvested cultures into the following media: PPLO agar, blood agar, thioglycollate broth, and Sabouraud's medium.

(2) Identify organisms detected in the above media by bacteriological and mycological methods.

V. Time schedules

a. Preparation of cell culture maintenance media

All lots of medium to be used on cell cultures that will be inoculated with crew material will be available 2 weeks before the lunar samples

arrive. The lots of medium will be tested for sterility and toxicity in the manner described under quality control work, sec. 3.3.3.2.1, part IV,b.

b. Cell cultures will be prepared from frozen stocks. Approximately 10 days will be required to obtain the cultures required for inoculation.

c. Cell cultures will be passed into the cabinetry 12 to 24 hours before they are used for virus isolation studies.

d. The cell cultures will be inoculated with samples as soon as the latter can be prepared for testing.

e. Cell cultures will be incubated at an appropriate temperature for a period of 7 days and subpassaged.

f. Cell cultures incubated for 14 days will be passaged.

VI. Pretest preparation of test area

a. Entry of equipment into Class III Cabinetry

(1) Autoclavable equipment (steam process)

(a) Place the equipment into the autoclave of the Class III Cabinetry in room 1-105.

(b) Autoclave the equipment at 121° C for 30 minutes and 15 psi pressure.

(c) Remove the equipment through the inside door and pass it to the rear arm of the Class III Cabinetry.

(2) Nonautoclavable equipment

Equipment which will be passed through Class III autoclaves (ethylene oxide gas process)

(a) Sterilize items with ethylene oxide gas in the autoclave attached to the Class III Cabinetry in room 1-105.

(b) Remove items through the inside door and pass to a preselected area of the Class III Cabinetry.

b. Standards to be met

(1) Class III Cabinetry

(a) Certify all Class III Cabinetry for 100-percent isolation.

(b) Sterilize all Class III Cabinetry and supply with sterile air.

(c) Sterilize all equipment and apparatus used within Class III Cabinetry.

(2) Sterilization apparatus

(a) Test all cabinetry autoclaves for the ability to kill Bacillus stearothermophilus spores.

(b) Test all cabinetry ethylene oxide gas sterilizers for the ability to kill Bacillus subtilis spores.

(3) Equipment and apparatus

(a) Steam-sterilize all autoclavable equipment in the cabinet autoclave for 30 minutes at 121° C and 15 psi pressure.

(b) Sterilize nonautoclavable material with ethylene oxide gas or pass it through the 5000-ppm sodium hypochlorite dunk tank.

c. Methods to be employed

Class III Cabinetry

(1) Certify all Class III Cabinetry as biological barriers by using Freon leak detectors (OPV7S2.8).

(2) Sterilize all Class III Cabinetry with paraformaldehyde according to procedures outlined in OPV7S2.14.

(3) Sterility test all Class III Cabinetry by swabbing and inoculating trypticase glucose yeast extract agar (OPV4S12.2.3) and Chanock, Hayflick, and Barile's Diphasic Mycoplasma medium.

(4) Monitor cabinet air by employing trypticase glucose yeast extract agar plates and Chanock, Hayflick, and Barile's Mycoplasma agar plates in Reynier air samplers operated at 1 cfm (OMV4S.14.6).

3.3.3.2.2 Embryonated eggs.-

I. Test design

Using 6- and 10-day-old embryonated eggs, two groups consisting of six eggs each will be inoculated with throat swabs or blood specimens by one of three routes as follows:

a. Group one (10-day-old eggs) — Amniotic sac-allantoic sac

b. Group two (6-day-old eggs) — Yolk sac

II. Test methods

a. Isolation systems

Embryonated eggs from white Leghorn chickens bred resistance-inducing factor-free will be used (Kimber Farms, Fremont, California, will be the supplier).

b. Entry of embryonated eggs into sterilized Class III Cabinetry for postflight analyses

(1) Immerse the eggs in a 1:256 solution of Vestal 1 Stroke Environ for 15 to 30 seconds.

(2) Place the eggs on sterile racks, and pass them into the cabinetry through autoclave.

c. Specimen requirements

(1) Pharyngeal swab

(2) Blood

d. Embryonated egg requirements

Isolation (initial) passage

(1) Pharyngeal swab — 18 eggs, 10 days old

Six eggs times one group times three astronauts

(2) Blood — 18 eggs, 6 days old

Six eggs times one group times three astronauts

(3) Controls — 12 eggs (six, 10 days old and six, 6 days old)

Six eggs time two groups

e. Preparation of specimen

Preparation of specimens is described in section 3.3.3.2.1, part II, a.

f. Inoculation

(1) Routes

(a) Amniotic sac-allantoic sac

(b) Yolk sac

(2) Volume

(a) Each egg inoculated via the amniotic-allantoic routes will receive 0.2 ml of sample.

(b) Each egg inoculated via the yolk sac route will receive 0.1 ml of sample.

(3) Preparation of eggs (according to route)

(a) Amniotic sac-allantoic sac

1 Candle the embryonated eggs and mark off the position of the embryo and the limit of the air sac with a pencil.

2 Punch a hole in the blunt end of the egg near the embryo (amniotic sac).

Punch the same eggs in the center of the blunt end of the egg (allantoic sac).

(b) Yolk sac

Prepare the eggs in the same manner as for amniotic sac inoculation.

(4) Introduction of inoculum (according to route)

(a) Amniotic sac

1 Introduce the sample into the sac with a 1-ml disposable syringe fitted with a 1-1/2-in. long, 20-gage needle.

2 Pass the needle through the hole in the blunt end of the egg and, with a quick motion, "hit" the amniotic sac with the needle (the embryo should jump).

3 Reclean the hole edges of the blunt end of the egg and seal with wax.

4 Place the needle and syringe into a disposal pan for autoclaving before disposal.

(b) Allantoic sac

Introduce the sample into the allantoic sac in the same manner as described under amniotic sac, except position the needle away from the embryo toward the shell.

(c) Yolk sac

Introduce the sample into the yolk sac in the same manner as described under amniotic sac, except position the needle directly toward the center of the egg.

(d) Controls

Six eggs will be inoculated with sterile diluent by the following routes:

1 Amniotic sac-allantoic sac

2 Yolk sac

g. Incubation

(1) Preinoculation

(a) Length of incubation (according to route)

1 Yolk sac — 6 days

2 Amniotic sac-allantoic sac — 10 days

(b) Conditions of incubation

Incubate the embryonated eggs with the blunt end up, at 38° C and at a 50- to 70-percent relatively humidity environment. Turn the eggs every 4 hours.

(2) Postinoculation

(a) Length of incubation (according to route)

Incubate the eggs inoculated via the amniotic-allantoic routes routinely for 96 hours. Incubate all eggs inoculated by the sac route for 6 days.

(b) Incubate the embryonated eggs with the blunt end up, at 36° C and at ambient humidity. Do not rotate the eggs.

(3) Scheme

(a) Nonspecific death of embryonated eggs

Discard embryonated eggs that die within the first 24 hours and do not count them in the final results. (Death occurring within the first 24 hours is usually a result of trauma or toxicity of the inoculum.)

(b) Carry out each of the three blind passages in the same manner. The number of eggs inoculated in each passage will remain constant.

h. Harvest

Chill all embryonated eggs for 12 to 18 hours at 4° C prior to harvest of fluids and membranes.

(1) Methods (according to route)

(a) Allantoic and amniotic fluids

1 Place the chilled egg in a support with the blunt end facing upward.

2 Clean the blunt end of the egg with a 1:256 solution of 1 Stroke Environ.

3 Break away the shell over the air sac and display the shell membrane.

4 Aspirate the allantoic fluid with a sterile 5-ml serological pipette and a propipette.

5 Pool the allantoic fluids from the five eggs that received the same inoculum.

6 Remove the fluids from the amniotic sac with a 1-ml syringe fitted with a 26-gage needle. (Flush out amniotic sacs with 1 to 2 ml of allantoic fluid harvest.)

7 Pool the amniotic fluids from the five eggs that received the same inoculum.

(b) Yolk sac

1 Prepare the chilled egg in the same manner as described under allantoic and amniotic fluids.

2 Loosen the CAM and decant the contents of the egg into a sterile Petri dish.

3 Detach the yolk sac from the embryo, rupture the sac, and allow it to drain.

4 Transfer the drained membranes to another sterile Petri dish and photograph.

(2) Fate of harvested fluids and membranes

(a) Fluids (amniotic and allantoic)

1 Test each pool with guinea pig and chick red blood cells for hemagglutinating particles.

2 Combine allantoic and amniotic pools for subpassage.

3 Store the remainder of each pool outside of the Class III Cabinetry at -70° C. The procedure for passing cultures to the -70° C freezer outside of the Class III Cabinetry is described in section 3.3.1.2.2, part II, k.

(b) Membranes (yolk sac)

1 Use one membrane of each inoculum (six eggs/inoculum) for histological examination.

Fix the tissues in alchol-formalin for 24 hours and remove them to the Histology Laboratory, employing procedures described in OPV7S2.13.

2 Use one membrane of each inoculum for ultramicroscopic examination. Prepare the membranes for electron microscopy as described in appendix C.

3 Using a sterile mortar and pestle, three membranes of each inoculum will be respectively pooled and ground with sterile Alundum and used for subpassage and/or storage at -70° C. The procedure will be as follows:

a Grind the three membranes thoroughly with sterile Alundum.

b Add 3 ml of tryptose phosphate broth with 0.5-percent gelatin and mix well.

c Sediment the suspension at 1500 rpm for 20 minutes.

d Remove the supernatant and use for subpassage and/or storage.

i. Observations

(1) Perform the following gross observations and photography of the eggs within the Class III Cabinetry.

(a) Death of the embryo (no movement is detected when the egg is candled)

(b) Retraction of blood vessels (retraction indicates the embryo is dead or dying)

(2) Histological examination of membranes

Examine fixed and stained membranes for the presence of intracellular viral inclusions as well as for pathological changes in the cells.

j. Backup studies

(1) Hemagglutination titer

(2) Effect on other systems

(a) Tissue culture

(b) Newborn and adult mice

k. Exit of materials from Class III Cabinetry during mission

(1) Autoclavable material

(a) Place the material to be steam autoclaved in the cabinet autocalve as per instructions in the Biological Safety Manual, Report Number MSC 00018.

(b) Remove the material through the outside door of the steam autoclave.

(2) Nonautoclavable material to be carboxyclaved

(a) Place materials to be sterilized in the cabinet autoclave and expose to ethylene oxide gas for a period of 16 hours.

(b) Remove the material through the outside door of the autoclave.

(3) Procedures for passing cultures to -70° C freezer outside of Class III Cabinetry

(a) Place the material to be stored at -70° C into double-stoppered Virtis vials.

(b) Pass the vials into the 5000-ppm sodium hypochlorite dunk tank for a 30-minute sterilizing time.

(c) Place the vials into metal storage containers and seal the containers with silicon stoppers.

(d) Place the containers in the -70° C freezer located in room 1-135.

1. Emergency plans

(1) Host failure

(a) A backup supply of 100 eggs (10-day old and 6-day old) will be available in the Support Laboratory at the time of each passage.

(b) A backup supply of 100 eggs (10-day old and 6-day old) will be available from the supplier at the time of each passage.

(2) Technician failure

Available personnel in the LRL Biological Sciences section will be cross-trained.

(3) Equipment failure

Duplicates of all equipment will be available in the Support Laboratories and will be transferred behind the barrier in case of failure.

III. Support effort

a. Materials required

(1) Embryonated eggs needed, isolation passage

(a) Pharyngeal swab

Six eggs times one group times three astronauts = 18 eggs, 10 days old

(b) Blood

Six eggs times one group times three astronauts = 18 eggs, 6 days old

(c) Control

Six eggs times two groups = 12 eggs (6 eggs, 10 days old, and 6 eggs, 6 days old)

Overage 10-day old	24 eggs
6-day old	24 eggs

(2) Embryonated eggs needed, blind passage

(a) Total eggs required/blind passage

10-day old	42 eggs
6-day old	42 eggs

(b) Embryonated eggs needed for three passages, including overages

10-day old	126 eggs
6-day old	126 eggs

IV. Quality control

a. Determination of the sterility of eggs

A representative sample (10 percent) of eggs will be tested for bacteria, fungi, and Mycoplasma with the following media: blood agar, PPLO agar, thioglycollate broth and Sabouraud's medium.

b. Each pool of harvested fluids and membranes of each blind passage will be tested for bacteria, fungi, and Mycoplasma in the following manner:

(1) Inoculate 0.1 ml of each pool into the following media: blood agar, PPLO agar, thioglycollate broth, and Sabouraud's medium.

(2) Organisms detected in the above media will be identified by bacteriological and mycological methods.

c. Microbiological status of the embryonated eggs will be determined by the supplier prior to shipment.

V. Time schedules

a. Blind passages

(1) Embryonated eggs inoculated by the amniotic-allantoic sac routes will be routinely incubated 4 days for each blind passage.

(2) Embryonated eggs inoculated by the yolk sac route will be routinely incubated 6 days for each blind passage.

b. Emergency pool

An aliquot of each pool of harvested fluid or membrane will by stored at -70° C outside of the Class III Cabinetry in case of nonviral contamination or for future reference. The procedure for passing cultures to the -70° C freezer outside of Class III Cabinetry is described in section 3.3.1.2.2, part II, j.3.

VI. Pretest preparation of Class III Cabinetry

The pretest preparation of Class III Cabinetry will be the same as that described in section 3.3.1.2.1, part V.

3.3.3.2.3 Suckling mice.-

I. Test design

Suckling mice, 24- to 48-hours old, will be inoculated with fecal and blood specimens via the intraperitoneal and intracerebral route.

II. Test method

a. Host

Pregnant albino mice having a known conception date will be obtained from a local supplier. These mice will be delivered to the laboratory 5 days prior to parturition. They will be maintained under ambient conditions in the support laboratory area until their litters are born.

b. Entry of mice into sterilized Class III Cabinetry for post-flight analyses

(1) The mother and her litter, in a freshly cleaned cage, will be passed into the Sample Laboratory through the airlock.

(2) They will be passed into the Class III Cabinetry in room 1-105 through a presterilized autoclave.

c. Crew specimen requirement

(1) Feces

(2) Blood

d. Mouse requirement

Isolation passage

(1) Feces

one litter times one specimen times three astronauts = three litters

(2) Blood

one litter times one specimen times three astronauts = three litters

(3) Inoculated control

one litter times two routes = two litters

(4) Noninoculated control = one litter

Each litter will contain not less than six nor more than ten mice.

e. Preparation of specimen

The preparation of the specimens will be the same as those described for tissue culture, section 3.3.3.2.1, part II, e.

f. Inoculation

Routes and volume

Inoculate each animal in a litter with 0.5 ml of specimen intraperitoneally and 0.02 ml of specimen intracerebrally. Use a 1-ml syringe and a 26-gage, 3/8-inch needle.

g. Incubation

Maintain the mice under ambient conditions within the Class III Cabinetry.

h. Detection of infection

(1) Examine the mice daily for signs of infection.

(2) Mice dying within the first 24 hours after inoculation are to be passed out of the Class III Cabinetry and into the -70° C freezer. These deaths are usually a result of trauma or toxicity of the inoculum. These specimens will be saved but not subpassaged unless justified by later analyses.

(3) Mice that develop paralysis, tremors, spasms, or which die more than 24 hours after inoculation will be autopsied and passaged.

(4) Gross observations will be made about the general condition of internal organs.

(5) Subcultivation of materials will be accomplished via the same route, and the same number of animals will be used in the primary isolation passage.

(6) Mice will be examined for 14 days following inoculation. Should no symptoms be observed at the end of that time, the animals will be sacrificed and subpassaged.

(7) One blind passage will be performed.

i. Harvest

(1) Sponge the external surface of the mouse with 70 percent alcohol.

(2) Secure the mouse, belly down, to a dissection board.

(3) Remove first the brain and then the entire skinned eviscerated torso and grind both with a sterile mortar and pestle.

j. Fate of harvested materials

(1) Make a 20-percent tissue suspension with phosphate-buffered saline and use this material for subpassage.

(2) Store an aliquot of the harvest at -70° C for further analyses.

k. Identification studies

(1) Mouse neutralization test

(2) Complement fixation

(3) Hemagglutination and hemagglutination-inhibition

l. Backup studies

Effect on other systems

(1) Tissue cultures

(2) Adult mice

m. Exit of materials from Class III Cabinetry during a mission.

Exit of materials from Class III Cabinetry during a mission will be as described in section 3.3.3.2.1, part II, m.

n. Emergency plans

(1) Host failure

(a) A 100-percent backup supply of aminals will be available in the Support Laboratory for immediate transport into the Class III Cabinetry.

(b) A 100-percent backup supply of animals will be available from the local supplier.

(2) Technician failure

Available personnel in the LRL Biological Sciences section will be cross-trained.

(3) Equipment failure

Duplicates of all equipment will be available in the support laboratories and will be transferred behind the barrier in case of failure.

III. Support effort

Materials required — litters of suckling mice (24- to 48-hours old).

Isolation passage	9 litters (54 to 108 mice)
Blind passage	9 litters (54 to 108 mice)
100-percent overage	18 litters
Total animals required	36 litters

IV. Quality control

a. All adult animals accepted for use in these studies will be examined for the presence of ectoparasites and other overt signs of possible ill health.

b. The presence of any signs of ill health in an adult mouse will result in its exclusion from testing.

V. Time schedules

a. Pregnant mice will be delivered to the laboratory 5 days prior to the expected date of parturition to allow adequate time for acclimatization and examination for signs of overt disease conditions.

b. All animals will be held and examined for a period of 14 days following inoculation prior to subpassage.

VI. Pretest preparation of Class III Cabinetry

The pretest preparation will be the same as that described in section 3.3.1.2.1, part V.

3.3.3.2.4 Mycoplasma.-

I. Test design

The modified medium of Chanock, Hayflick, and Barile will be employed at pH 6.3 and 7.8 in

diphasic and agar forms in an attempt to isolate mycoplasma organisms from all crew specimens.

II. Test method

a. Isolation system

The medium of Chanock, Hayflick, and Barile is used in the following modifications:

(1) Agamma horse serum will be used.

(2) Eagles's vitamins (final concentration, 1.0 percent), arginine (10 mM) will be added.

(3) Glucose (final concentration 0.2 percent) will be added.

(4) Thallium acetate (1:1000 dilution) and penicillin (1000 units/ml) will be added.

b. Entry of mycoplasma media into the Class III Cabinetry

(1) Prepare all media in the Support Laboratory.

(2) Pass media into the cabinetry through the autoclave.

c. Specimen requirement

(1) Feces

(2) Urine

(3) Pharyngeal swab

(4) Blood

d. Medium requirements

(1) Diphasic bottles (isolation passage)

two bottles times four specimens times three astronauts = 24 bottles

(2) Agar plates

Two plates times four specimens times three astronauts = 24 plates

e. Preparation of specimen

Prepare the specimens as described in section 3.3.3.2.1, part II, e.

f. Isolation

(1) Inoculation

(a) Each diphasic bottle will receive 0.2 ml of inoculum.

(b) Each group of bottles inoculated with the same specimen will be labeled with the name and date.

(2) Incubation temperature and environment

Incubate the bottles at 36° C. Incubate the agar plates at 36° C under 70- to 80-percent humidity and 5.0-percent CO_2 in nitrogen.

(3) Blind passages

(a) Two-tenths milliliter of the broth from diphasic bottles will be inoculated into fresh bottles and plates every 3 days.

(b) Agar plates will be held for a total of 2 weeks and examined daily for colonies.

(c) A total of two blind passages of diphasic bottles will be conducted.

g. Observations

The inoculated media will be examined daily for evidence of growth.

(1) Examine broth phase of the diphasic cultures for turbidity and/or change in the pH of the medium.

(2) Examine the agar plates daily with a microscope mounted in the Class III Cabinetry in room 1-105.

(3) The presence of microcolonies which usually have the typical "fried egg" appearance may indicate the presence of mycoplasma organisms.

h. Backup studies

(1) Hemolysis, employing human, chicken, guinea pig, sheep, rabbit and horse red blood cells (RBC)

(2) Hemadsorption, employing the above RBC's

(3) Growth inhibition by specific antisera

(4) Complement fixation

(5) Gel-diffusion

(6) Indirect hemagglutination

(7) Reaction of colonies with Diene's stain

(8) Metabolic inhibition

i. Exit of materials from Class III Cabinetry

Exit of materials from Class III Cabinetry will be performed as described in section 3.3.3.2.1, part II, m.

j. Emergency plans

(1) Isolation system failure

A 100-percent backup supply of pretested medium will be available in the Support Laboratory.

(2) Technician failure

Available personnel in the LRL Biological Sciences Section will be cross-trained.

(3) Equipment failure

Duplicates of all equipment will be available in the Support Laboratories and will be transferred behind the barrier in case of failure.

III. Support effort

a. Isolation passage

Medium required

Two bottles times four specimens times three astronauts = 24 bottles

Two plates times four specimens times three astronauts = 24 plates

Overage 24 bottles

24 plates

b. Blind passages

Two bottles times four specimens times three astronauts times two passages = 48 bottles

Two plates times four specimens times three astronauts times two passages = 48 plates

Overage 48 bottles

48 plates

Total medium requires 144 diphasic bottles

144 agar plates

IV. Quality control work

a. Medium

(1) Chanock, Hayflick, Barile's medium, prepared in this laboratory, will be tested for its ability to support the growth of the following mycoplasma strains: M. pneumoniae (Eaton's agent) and T strains.

(2) All complete medium will be tested for sterility by incubation at 35° C for 18 hours in the Class III Cabinetry.

(3) Each lot of agamma horse serum and vitamin-yeast extract will be tested for sterility 2 weeks before receipt of the sample.

b. All pipettes and glassware will be washed in a biodegradable soap and rinsed in demineralized and glass-distilled water..

V. Pretest preparation of Class III Cabinetry

The pretest preparation of the test area will be the same as described in section 3.3.1.2.1, part V.

APPENDIX A

FLOW DIAGRAMS

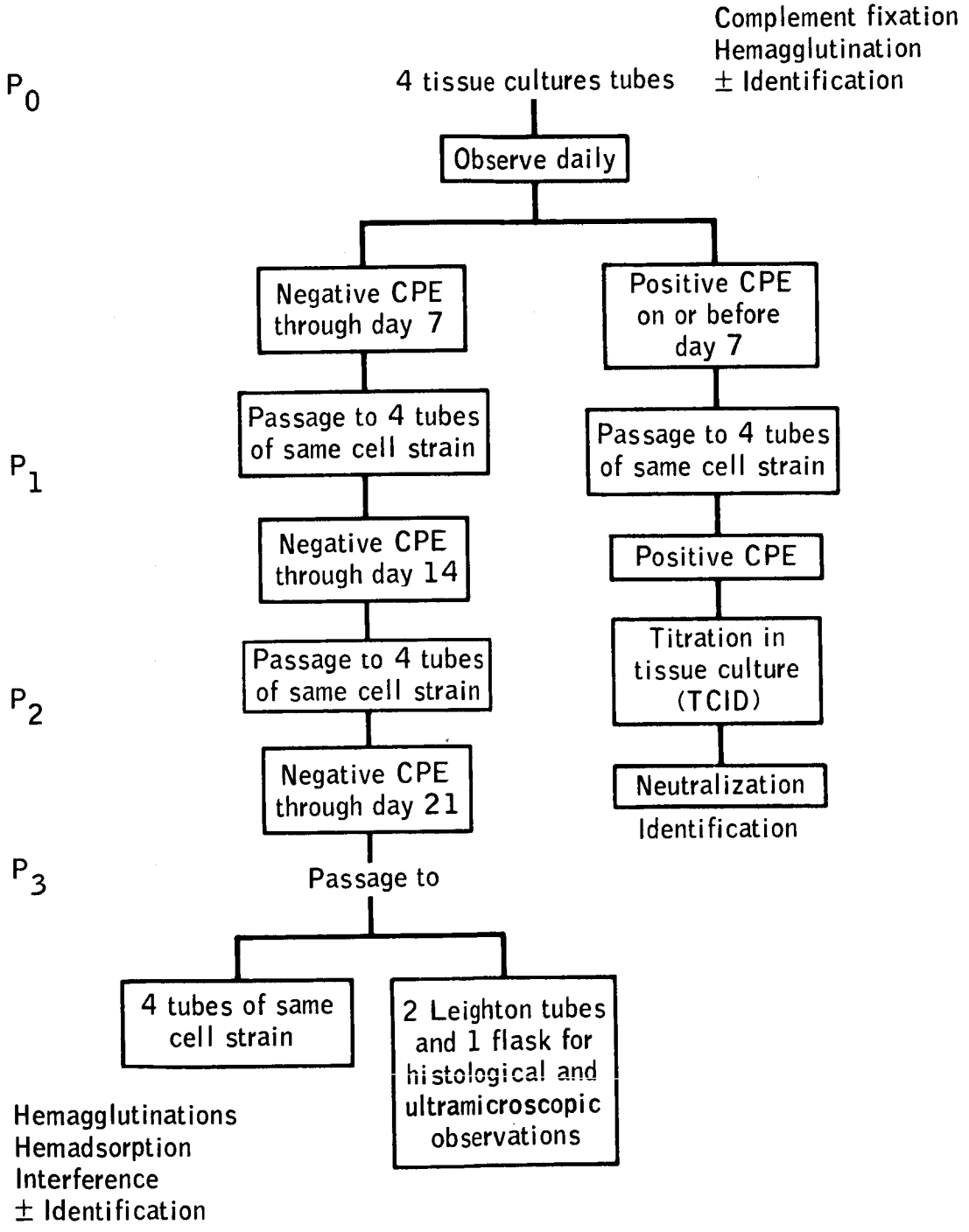


Figure A-1.- Detection of infection (rapidly growing agents).

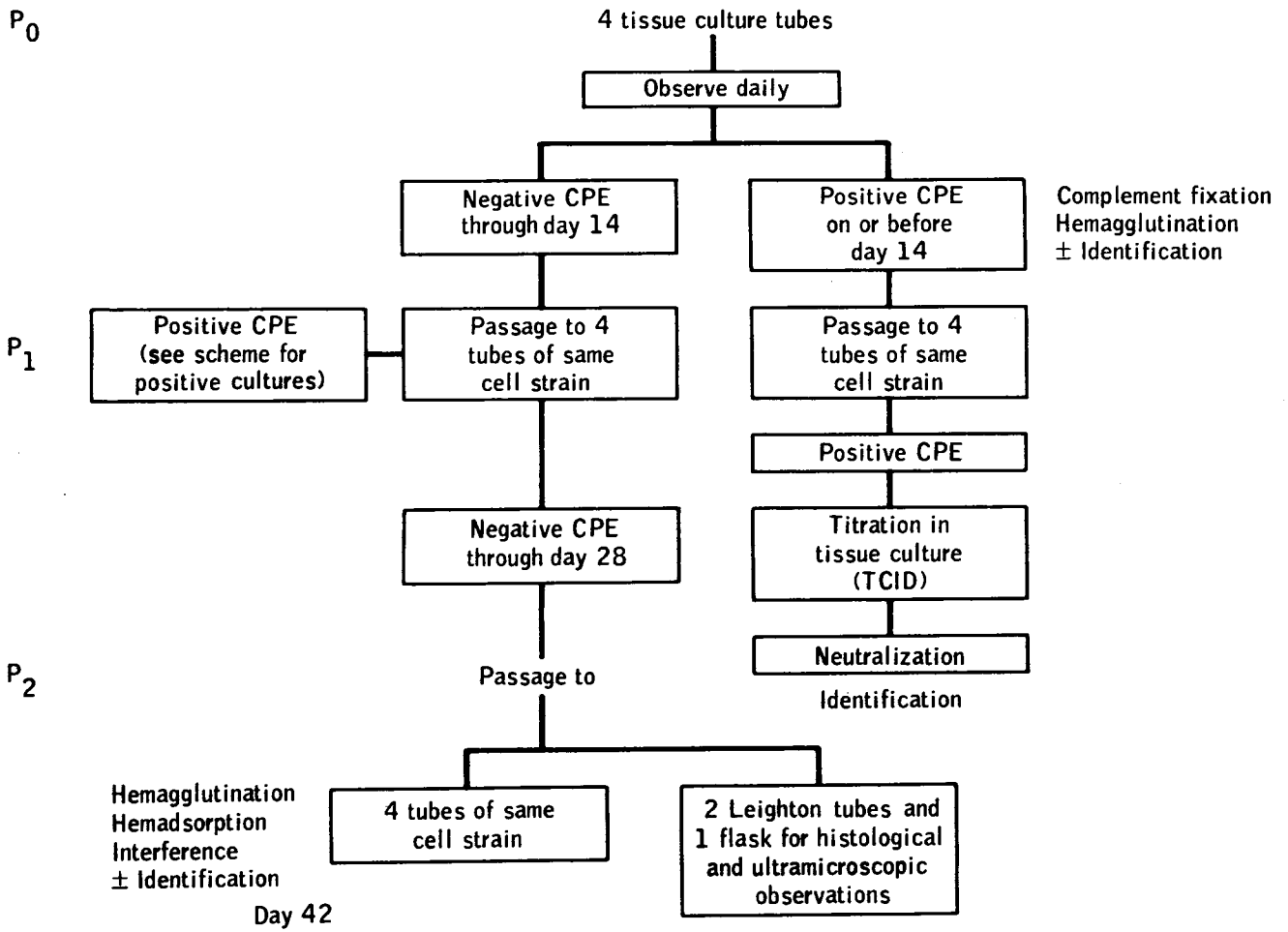


Figure A-2.- Detection of infection (slowly growing agent).

APPENDIX B

STAINS

I. Hematoxylin and eosin stain for Leighton tubes

a. Procedure

(1) Remove the cover slips from the tubes, and place the cover slips in a carriage.

(2) Wash in glass-distilled water.

(3) Fix in absolute alcohol for a minimum of 15 minutes.

(4) Rinse in distilled water.

(5) Immerse twice in absolute alcohol.

(6) Immerse twice in 95 percent alcohol.

(7) Immerse twice in 70 percent alcohol.

(8) Rinse in distilled water.

(9) Stain in hematoxylin for 2 and 1/2 minutes.

(10) Rinse in glass-distilled water.

(11) Immerse twice in acid alcohol.

(12) Rinse in distilled water.

(13) Immerse twice in ammonia water.

(14) Rinse in distilled water.

(15) Immerse in 70 percent alcohol for 1 minute.

(16) Stain in 1 percent eosin for 3 minutes.

(17) Immerse in 95 percent alcohol for 1 minute.

(18) Immerse in 100 percent alcohol for 3 minutes.

(19) Stain in xylene for 5 minutes.

(20) Mount with permount (ref.1).

b. Results

(1) The nuclear structures retain the basic dye of hematoxylin and remain blue.

(2) The cytoplasm stains red with the eosin.

II. Safranin stain for Leighton tubes

a. Procedure

(1) Remove the cover slips from the tubes, and place the cover slips in a carriage.

(2) Rinse in glass-distilled water.

(3) Rinse three to five times in absolute alcohol.

(4) Immerse twice in 95 percent alcohol.

(5) Immerse twice in 70 percent alcohol.

(6) Rinse in distilled water.

(7) Stain in Babés' solution for 1 to 2 minutes.

(8) Rinse in glass-distilled water.

(9) Differentiate in 95 percent alcohol.

(10) Repeat in 100 percent alcohol for 5 minutes.

(11) Stain in xylene for 5 minutes.

(12) Mount with permount.

b. Results

(1) The nuclei stain light red.

(2) The mitotic figures stain intense red.

(3) The background is colorless or pale pink (ref.1).

III. Diene's stain for pleuropneumonia-like organisms (stained agar technique)

a. Procedure

Make the staining solution by dissolving 2.5 g methylene blue, 1.25 g azure II, 10 g maltose, 0.25 g $Na_2 CO_3$, and 0.2 g benzoic acid in 100-ml distilled water. With a cotton applicator, apply a thin film of staining solution to cover slips (24- by 24-mm no. 1) and allow it to dry. Cut the cover slips into four squares using a diamond-point pencil. Cut blocks of agar (about 6 to 8 mm) out of the culture and place right side up on a glass slide. Place the dye-coated cover slips stain side down, directly on the agar blocks so that a projecting rim of cover slip extends over all sides of the agar. Fill the space between the cover slips and the slide with melted paraffin containing 10-percent Vaseline which, upon hardening, seals the preparation and permits examination under oil-immersion without disturbing the cover slip.

b. Results

Staining is completed within a few minutes. Colonies of PPLO stain bright blue as do young viable bacterial colonies. Autolyzed bacteria, cellular material, and debris take on a pink hue. The agar stains faintly blue or violet. If the agar stains too deeply, it may be necessary to dilute the stain somewhat. If the agar is thicker than 2 to 3 mm and not transparent, it may be necessary to examine a thin layer sliced from the top of the block by means of a razor blade. Even chocolate or blood agar may be treated in this way to give a satisfactory preparation. In selecting the block of agar to be examined, avoid large bacterial colonies whenever possible, as they tend to decolorize the stain locally. This difficulty usually can be avoided by a brief exposure (approximately 20 seconds) of the block to the fumes of a loopful of concentrated HCl. This is done in a small Petri dish before staining. It is important to examine several preparations from a single

culture from both heavily and lightly inoculated areas as well as from areas adjacent to bacterial colonies (ref. 2).

APPENDIX C

PREPARATION OF MATERIAL FOR EXAMINATION WITH THE ELECTRON MICROSCOPE

I. Types of materials

Pellets from cell cultures and membranes from embryonated eggs are processed by these same procedures. In this appendix, the word pellet is used to apply to both pellets and membranes.

II. Procedures

a. Preparation of the Epon block

(1) Transfer the material received from the Sample Laboratory to a screwcap tube containing 10 ml of 0.1M phosphate buffer (pH 7).

(2) Centrifuge the material at 1500 rpm at 4° C for 10 minutes. Repeat this process three times or until no foreign material can be seen adhering to the pellet. Discard all wash fluids.

(3) Use 2 ml of 2 percent osmic acid in phosphate buffer to fix the pellet for 1 hour. Decant the osmic acid buffer.

(4) Wash the pellet in phosphate buffer for 15 minutes. Repeat the wash one time.

(5) Dehydrate the pellet (or membrane) in successive 2-ml baths of 50, 70, 95, and 100 percent ethyl alcohol. The contact period of each bath will be 20 minutes. Repeat the 100 percent ethyl alcohol bath one time. Discard each bath.

(6) Dehydrate the pellet in 2 ml of propylene oxide for 15 minutes. Repeat the process once. Discard the propylene oxide.

(7) Remove the pellet with a spatula and transfer to an 8-dram vial containing 2 ml of a 1:1 mixture of Epon 812 and propylene oxide with 3 percent butyl-dimethyl aniline (BDMA). Use a contact period of 2 hours.

(8) Add an equal portion of 3 percent BDMA and mix. Use a contact period of 14 hours (overnight).

(9) Prepare fresh Epon with 3 percent BDMA. Add one drop of the mixture to each Beem capsule. Place the pellets in approximately 1-cu mm pieces, in each capsule, and add the fresh Epon until the capsules have been filled.

(10) Allow the Epon mixture to harden in a dry-heat oven at 60° C.

(11) After the Epon has hardened, remove the capsules from the oven. Section or store the capsules after they have cooled to room temperature.

(12) Break off the lid of the Beem capsule.

(13) Using a single-edge razor blade, cut the remaining capsule into thirds along the length of the capsule. Peel the sections backward in order to separate the Epon block from the capsule shell.

b. Cutting and mounting tissue sections

(1) Insert the Epon block into the collet-type holder of the Sorvall ultramicrotome so that the tissue end of the Epon block is 3/16 inch from the front face of the holder; tighten in this position.

(2) Mount the collet-type holder containing the Epon block in the trimming block.

(3) Fit the trimming block to the microtome table by raising the knife-stage rotation lock and sliding the block onto the table so that the small turret that projects from the table will engage the slot in the block. Leave the lock loose to allow the block to rotate as the specimen is trimmed.

(4) Use a single-edge razor blade to shape the Epon block into a truncated pyramid with sides of approximately 1 mm.

(5) Trim the face of the block to a trapezoid configuration with parallel top and bottom edges and sloping sides. Make the sections from just under the front face of the block. In general, the block face will be no larger than 1-mm square.

(6) Remove the collet-type holder from the trimming block and mount it on the end of the cantilever arm. The holder is held in place by the rotation-locking-ring thread in the rear of the specimen-holder mount. Finger tighten and then lock using the tool provided.

(7) Insert the diamond knife or the glass knife into its holder.

(8) Insert the knife holder into the knife-stage assembly by raising the locking lever and sliding the holder in from the rear into its slot. Set the holder at the desired clearance angle referring to the scale (graduated in 2° divisions) on the right side of the holder. Lock the holder in place by downward pressure on the locking lever. The best clearance angle is usually at 3° to 5°.

(9) Activate the microtome-advance system by first depressing the RESET button, then depressing the MOTOR button. Set the upper thickness control to assure a desired setting of 10.

(10) Next, depress the OFF button and rotate the hand wheel in a clockwise direction through at least one complete cycle; stop with the block slightly above its midpoint in the downward cycle so that the cantilever arm will be in its forward position.

(11) Loosen the knife-advance and lateral-movement lock. Bring the knife to within 1 mm of the specimen and aline it with respect to the block. Tighten both

the knife-advance and lateral-movement lock.

(12) Loosen the fine advance-engagement screw so that the knife advance will be in its coarse position, allowing the knife to be advanced so that a small space is left between the specimen and the knife edge.

(13) Tighten the fine advance-engagement screw to allow the forward advance to be in its fine position. Unlock the knife-stage rotation lock at the left side of the table by raising it against the stop. The stage will be rotated until the knife appears to be parallel to the front face of the block. Lock the knife stage in this position by pressing down on the knife-stage rotation lever.

(14) Fill the knife trough with distilled water.

(15) Depress the ON button. Make adjustment of the tissue thickness to about 600 Å or less. Make adjustment of the speed to about 0.35 mm/sec.

(16) Cut and float six to ten tissue sections into the knife trough. Pick up the sections on the carbon side of a 3.2-mm grid.

(17) Blot the grid dry by touching its edge to filter paper.

(18) Place the grid carbon side down on a drop of 50-percent uranyl acetate and 50 percent distilled water mixture for 10 minutes.

(19) Wash the grid by dipping it into distilled water.

(20) Place the grid carbon side down in a lead citrate solution for 2 minutes.

(21) Wash the grid in successive baths of a 0.025N solution of sodium hydroxide and distilled water. Blot the grid dry by touching its edge to filter paper. Insert the grid into the electron microscope.

(22) Examine three grids for each pellet. Survey 200 holes within each grid. Examine 50 fields per hole (at 20 000X power).

(23) Take photographs of fields showing evidence of microbial life of any type or changes in cell structure caused by microbial replication.

APPENDIX D

MEDIA

I. PPLO medium number 1, Eaton agent agar medium

a. Ingredients

(1) Agar (70 percent)

(a) 34 g Difco PPLO agar

(b) 1000 ml of distilled water

(c) Mix and heat to boiling in flask until clear

(d) When agar is dissolved, dispense 70-ml portions into 4-oz prescription bottles

(e) Autoclave at 15 psi pressure and 121° C for 15 minutes

(f) Store at 4° C for no longer than 3 months

(2) Serum (20 percent)

Sterile, agamma horse serum

(a) Filter, if necessary, to remove precipitate

(b) Dispense in 20-ml portions in sterile tubes

(c) Label, date, and store at 4° C

(3) Vitamins (Eagle's), arginine (1.15 mM), glutamine (1.4 mM), and Glucose (0.2 percent)

Stock solution (100X)

(a)	Choline	5.0 mg
(b)	Folic acid	5.0 mg

(c)	Inositol	10.0 mg
(d)	Nicotinamide	5.0 mg
(e)	Pantothenate	5.0 mg
(f)	Pyridoxal	5.0 mg
(g)	Riboflavin	0.5 mg
(h)	Thiamine	5.0 mg
(i)	Arginine	1.0 g
(j)	Glutamine	1.0 g
(k)	Glucose	20.0 g

(l) Dissolve in distilled water and bring up to 50 ml.

(m) Add to yeast extract solution before final filtration. (1 ml of the 100X solution, etc., for every 10 ml of 25 percent yeast extract solution.)

(4) Yeast extract (10 percent) of a 25-percent solution

(a) 125 g of Fleischmann's active dry baker's yeast.

(b) 500 ml of distilled water.

(c) Heat water to a temperature between 42° and 45° C in a large beaker with magnetic stirrer. Stirrer should be off.

(d) Add yeast slowly and cover. Allow to soak without stirring for 5 minutes.

(e) Stir until dissolved.

(f) Boil for 5 minutes.

(g) Centrifuge yeast extract for 20 minutes at 2000 rpm (1000 times gravity) and save clear supernatant.

(h) Add vitamins 100X solution. One ml for each 10 ml of yeast extract solution.

(i) If necessary, adjust pH to 8.0 with 1N NaOH on pH meter.

(j) Filter through Whatman number 1 filter paper.

(k) Filter through sterilizing filter.

(l) Dispense 11 ml per sterile screwcap tube.

(m) Freeze on slant and store at -20° C.

b. Preparation for use

(1) Melt one bottle of agar (20 ml) in boiling water.

(2) Transfer agar to 45° C water bath.

(3) Thaw one yeast-extract vitamin tube (11 ml) in 37° C water bath.

(4) Place one tube of agamma horse serum (20 ml) in 37° C water bath.

(5) When all three ingredients have warmed, combine, using sterile technique.

(6) Fill sterile plastic Petri dishes (60 mm) approximately one-third full.

(7) When medium is cool and solidified, store at 4° C for no longer than 3 weeks.

II. PPLO medium number 2, Eaton agent broth medium

See medium number 1, Eaton agent agar, for supplemental ingredients.

a. Ingredients

Broth

(1) 21 g of Difco PPLO broth without crystal violet

(2) 1000 ml of distilled water

(3) Mix and stir until dissolved

(4) Dispense 70-ml portions into 4-oz prescription bottles

(5) Autoclave at 15 pounds of pressure and 121° C for 15 minutes

(6) Label, date, and store at 4° C (up to 3 months)

b. Preparation

(1) Combine 70 ml of broth, 20 ml of agamma horse serum and 11 ml of vitamin-yeast extract, and dispense in 3- to 5-ml amounts in sterile screwcap tubes

(2) Label, date, and store at 4° C (up to 3 weeks)

APPENDIX E

IDENTIFICATION OF MYCOPLASMA SPECIES

I. Identification of mycoplasma strains by hemolysin production (ref. 6)

a. Erythrocytes

Collect horse, guinea pig, rabbit, and human blood samples in citrate saline solution. Wash the red cells three times in 0.85 percent saline. Add the erythrocytes to Difco PPLO agar medium (55° to 60° C) to give a final concentration of 4 percent blood cells. Pour approximately 3 ml of the erythrocyte-agar mixture as an overlay onto agar plates which contain mycoplasma colonies growing on the surface.

b. Test for hemolysin

Incubate the agar plates at 36° C for a minimum of 4 days. Observe the plates daily for hemolysis in the region of the mycoplasma colonies.

c. Interpretation

M. pneumoniae will produce Beta hemolysis with guinea pig and sheep cells and Alpha hemolysis with horse, human, and rabbit cells.

II. Identification of Mycoplasma pneumoniae by hemadsorption (ref. 7)

a. Preparation of red blood cells

Use red blood cells from humans, rabbits, guinea pig, horses, or sheep. Wash the cells three times in phosphate-buffered saline (pH 7.4) and prepare a 0.5-percent suspension.

b. Test

Flood the surface of agar plates on which are growing colonies of mycoplasma with the 0.5-percent suspension of red blood cells. Incubate the plates at 37° C for 30 minutes. Pour off the suspension of red blood cells and wash the surface of the agar

plate with phosphate-buffered saline. Examine the surface of the plates microscopically for hemadsorption of red blood cells.

III. Growth inhibition test for identification of mycoplasma species (ref. 8)

a. Impregnate sterile filter-paper discs, 6.35-mm diameter, with 0.02 ml of undiluted antiserum. Dry the discs for 3 days in a Petri dish at 5° C over anhydrous calcium chloride and silica gel.

b. Store the discs at -20° C until needed.

c. Inoculate agar plates (made with Chanock, Hayflick, and Barile's medium) with the test organisms by the push-block method.

d. Place the serum-impregnated discs on the surface of the agar plates.

e. Incubate the agar plates at 35° C in a humidified environment (80 percent) under 5 percent carbon dioxide and 95 percent nitrogen for approximately 4 days.

f. Examine the surface area adjacent to each disc microscopically for zones of inhibition.

IV. Identification of mycoplasma species by gel-diffusion (refs. 9 and 10)

a. Preparation of antigen

The mycoplasma under test will be grown for 2 to 4 days at 35° C in a diphasic culture bottle.

The organisms will be harvested by centrifugation at 10 000 times gravity for 80 minutes at 4° C, washed twice in phosphate-buffered saline (PBS), pH 7.2, and finally suspended in PBS to a concentration 100 times that of the original culture.

The suspension will be treated for 10 minutes with an ultrasonicator at 20 kHz.

b. Production of hyperimmune antisera

Antisera will be induced in rabbits with antigen plus Freund's adjuvant. The adjuvant will consist of a mixture of 9 parts Drakeol 6-VR and 1 part Arlacel. Mycoplasma species grown in 500 ml of culture medium for 3 to 4 days will be harvested by centrifugation at 10 000 times gravity, washed two times, resuspended in 2 ml of PBS, and homogenized with an equal volume of adjuvant.

Each rabbit will be inoculated subcutaneously in the flank with two injections (separated by an interval of 3 weeks). The injections will be followed by a course of six intravenous injections (separated by intervals of 1 week) 3 weeks after the last subcutaneous injection.

The antisera will be collected 5 to 7 days after the last intravenous injection.

c. Double-diffusion test

(1) A base layer of 1 percent agar will be poured into a Petri dish and allowed to harden.

(2) Molds will be placed on the surface of the agar in the desired pattern.

(3) A second layer of agar will be poured and allowed to harden.

(4) The molds will be carefully removed after the agar has hardened.

(5) The antigen and antisera will be added to the well in the agar.

(6) Precipitation will be allowed to take place at room temperature.

(7) Optimal development of bands of precipitate will be seen in 2 to 5 days depending on the antisera.

V. Identification of mycoplasma strains by indirect hemagglutination (ref. 11)

a. Tanning of sheep cells

Sheep erythrocytes in Alsever's solution will be washed three times with phosphate-buffered saline adjusted to a final concentration of 2.5 percent and mixed with an equal volume of a freshly prepared solution of 1:20 000 tannic acid in PBS. After 10 minutes incubation at 37° C, the cells will be centrifuged, washed once in PBS, and resuspended to 2.5 percent in PBS at pH 6.4.

b. Antigen preparation

A 100-ml broth culture of the mycoplasma species will be centrifuged at 30 000 rpm for 1 hour and the pellet resuspended in a volume of PBS (pH 7.2) equal to one-half that of the original broth. Centrifugation will be repeated under the same conditions and the sediment resuspended in buffered saline to give a final volume equal to 1/20 that of the original broth. The antigens will be treated in a 9KC Raytheon sonic oscillator for 10 minutes.

c. Sensitization of tanned sheep cells

One volume of antigen will be mixed with one volume of the 2.5-percent suspension of tannic acid treated cells in four volumes of PBS, pH 6.4, and the mixture will be incubated for 30 minutes at room temperature. The cells will be washed twice in rabbit serum diluent (heat-inactivated normal rabbit serum which has been absorbed with 50 percent sheep cells and diluted 1:150 in PBS) and adjusted to a 0.5-percent suspension in the same diluent.

d. Preparation of serum

The known sera will be inactivated at 56° C for 30 minutes, diluted 1:10 in rabbit serum diluent, and adsorbed at 4° C for 30 minutes using 0.1 ml of 50 percent erythrocytes per ml of diluted serum.

e. Test

The treated sera will be titrated in twofold dilutions using 0.4 ml of rabbit serum diluent in 13- by 100-mm tubes and an equal volume of a 0.5-percent suspension of sensitized erythrocytes will be added. The test will be incubated at 35° C, and read for hemagglutination when controls show well formed buttons (usually after 2 to 3 hours).

f. Interpretation

The reciprocal of the highest initial dilution yielding a clearly positive agglutination of the sensitized erythrocytes will be considered the end point (ref. 12).

VI. Mycoplasma metabolic inhibition test

a. Reagents

(1) One percent glucose broth with 0.002 percent phenol red, pH 7.8, or 1 percent arginine broth with 0.002 percent phenol red, pH 7.8

(2) Mycoplasma pneumoniae suspension

(3) Acute serum

(4) Convalescent serum

(5) Known positive serum

(6) Known negative serum

b. Procedures

(1) Label a microtiter U-plate as indicated in the plate pattern (fig. E-1) and add 0.025 ml of broth medium to each test well (except the first well in each serum row).

(2) Inactivate all sera at 56° C for 30 minutes.

(3) Add 0.05 ml of serum to the first well of the indicated row.

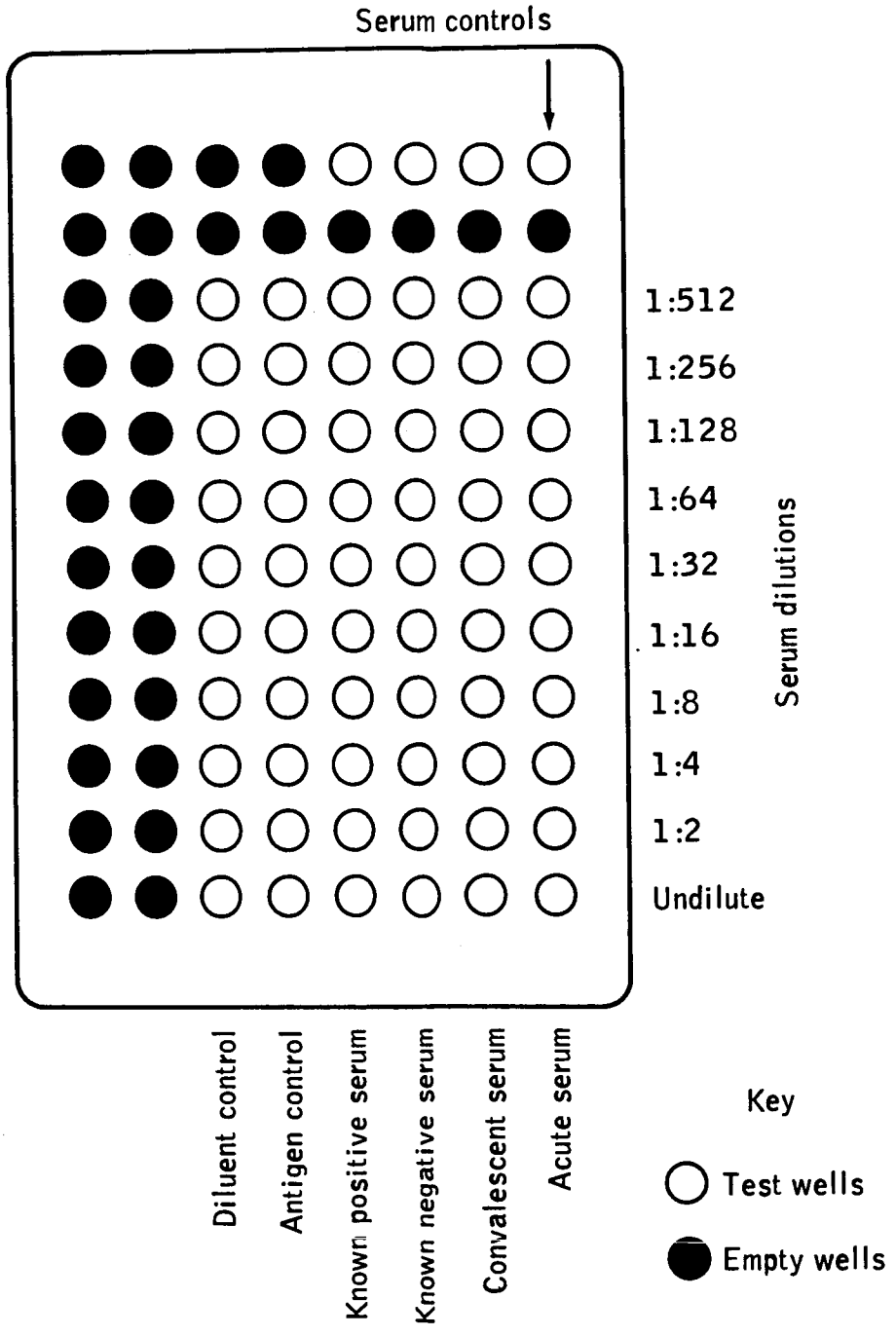


Figure E-1.- Plate for Mycoplasma pneumonia.

(4) Prepare twofold serial dilutions of each serum with a 0.025-ml microtiter loop.

(5) Add 0.05 ml of a mycoplasma suspension diluted to yield 10^3 to 10^4 organisms to each serum dilution (undilute through 1:512) and to the antigen control wells.

(6) Add 0.125 ml of glucose broth (5 drops with the 0.025-ml microtiter dropper) to each serum dilution (undilute through 1:512) and to the antigen control wells.

(7) Add 0.175 ml of glucose broth (7 drops with 0.025-ml microtiter dropper) to each diluent control well.

(8) Prepare the serum control wells by adding 0.025 ml of the appropriate serum and 0.150 ml of glucose broth.

(9) Seal the plate with cellophane tape and incubate at 37° C without agitation.

(10) Read the test over a mirror with a fluorescent light source daily for pH change.

c. Interpretation

Metabolic inhibition titers are defined as the highest dilution of serum that will cause 50 percent or greater inhibition of pH change (less than 0.25-pH-unit change when the mycoplasma controls have changed 0.5-pH unit).

ADDENDUM

Analysis of Effects of Lunar Material on Fish Tissue Cultures

I. Test design

a. Three types of fish cell cultures will be exposed to pooled lunar sample in an attempt to isolate agents capable of multiplication in these culture systems.

b. The sample will be introduced into the host cell cultures as the supernatant from a 50-percent suspension of lunar material in tryptose phosphate broth containing 0.5 percent gelatin. Any agent that is detected will be propagated and studied, and its identification will be attempted.

II. Test method

a. Host

Use the following cell lines:

(1) Rainbow trout gonadal tissue (RTG-2)
(2) Fathead minnow (FHM)
(3) Grunt fin (GF)

b. Sample requirement

(1) Infective dose

Use 25-cm^2 and 75-cm^2 Falcon flasks as culture vessels. The cell sheet area of a 25-cm^2 flask is comparable to four culture tubes and the cell sheet area of a 75-cm^2 flask is comparable to 12 culture tubes. Introduce the extract from 0.892 g of lunar material into each 25-cm^2 flask and introduce the extract from 2.676 g of lunar material into each 75-cm^2 flask.

(2) Pooled sample

(a) Sample — one

(b) Inocula — two

1 Supernatant from 50-percent suspension of soil

2 Supernatant from 50-percent suspension of sterile soil

(c) Medium — one

Antibiotic

(d) Cell culture containers

1 Falcon flasks, 25 cm^2 — two flasks/cell type

2 Falcon flasks, 75 cm^2 — one flask/cell type

(e) Number of cultures

1 Falcon flask cultures, 25 cm^2— 12 cell cultures

Two cell cultures/cell type times three cell types times two inocula times one medium times one sample

2 Falcon flask cultures, 75 cm^2— 6 cell cultures

One cell culture/cell type times three cell types times two inocula times one medium times one sample

(f) Amount of sample

1 For 25-cm^2 flask cultures 10.704 g

Twelve cell cultures times 0.892 g of sample

2 For 75-cm^2 flask cultures 16.056 g

Six cell cultures times 2.676 g sample

3 Total sample required 26.760 g

c. Preparation of sample for inoculation.

The sample requirement for the fish cell cultures will be incorporated into the sample requirement for all of the tissue cultures (see section 3.3.1.2.1, part II, c). The sample will be suspended and centrifuged. The necessary amount of supernatant, 26.0 ml, will be provided for the inoculation of the fish cell cultures.

d. Inoculation

Inoculate each 25-cm^2 culture flask with 0.8 ml of the supernatant from the sort suspension, and each 75-cm^2 culture flask with 2.4 ml of the same supernatant. Two hours after inoculation, examine the tissue cultures and make any required pH adjustments.

e. Incubation

(1) Temperature

Incubate the cultures at 15° C in the low-temperature bath-type incubation.

(2) Length of incubation

Incubate the cultures for 20 days.

(3) Scheme

(a) Observe the cell cultures daily, by direct microscopic observation, for evidence of infection. Take photographs of a random number of negative cultures at each observation.

(b) If no evidence of infection is present after 10 days, challenge two 25-cm^2 flasks of each cell line with 20 to 50 plaque-forming units (PFU) or 100 $TCID_{50}$ of ATCC strain VR229 infectious pancreatic necrosis (IPN) virus. Incubate the fluid from inoculated, but unaffected, cultures and from control cultures with appropriate amount of reference IPN virus for 30 minutes at 15° C. If the medium from the unaffected culture does not reduce PFU by 50 percent or more, it will be considered indicative of a lack of interferon.

(c) A reduction in $TCID_{50}$ may be substituted for plaque reduction in determining the presence of interferon.

(d) If no evidence of infection is present afer 20 days, harvest the cells from the 75-cm^2 flask of each cell culture for electron microscopy study (Sec. 3.3.1.2.1, part II, f. 3e6).

f. Observations

Determine the cause of effects in any of the cell lines. Call the CPE to the immediate attention of the on-site Bureau of Sport Fisheries and Wildlife Specialist and to Dr. Wolf of Eastern Fish Disease Laboratory.

g. Cell culture requirements

(1) Falcon flask cultures, $25cm^2$

Test cultures	12 cultures
Controls	12 cultures
Overage	30 cultures
Total	52 cultures

(2) Falcon flask cultures, $75cm^2$

Test cultures	6 cultures
Controls	6 cultures
Overage	3 cultures
Total	15 cultures

h. Emergency plans

The Eastern Fish Disease Laboratory has on hand and will maintain a backup of active cultures.

i. Sample clearance

Lack of effect in two of the three cell lines will be considered a basis for sample clearance.

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